

Catarina Sofia Rodrigues Fernandes

**Linking the embryonic clock with
temporal collinearity of *Hox* gene
activation**

LISBOA

2010

UNIVERSIDADE NOVA DE LISBOA

FACULDADE DE CIÊNCIAS E TECNOLOGIA

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*Dissertação apresentada para a obtenção do Grau de Mestre
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Orientadora:

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I. ACKNOWLEDGMENTS

There are many people that helped me in this journey throughout my MSc that I wish to thank.

Firstly, to my supervisor, Prof. Isabel Palmeirim, who has given me the opportunity to work in her laboratory and to be part of her new project. I thank you for your transmission of knowledge, life experience and advices at the right moments.

To my second supervisor, Raquel Andrade, for bringing me in and making me feel at home from the beginning. For her helpful knowledge, inner strength, trust and friendship throughout the year.

To Mónica Ferreira for the teachings of the strenuous electroporation methodology.

To Manuela Carneiro for the patience and sympathy shown in face of so many pieces of paper to autoclave.

To Margaret Soares for encouraging me to find the strength to not give up, and for the availability shown on helping me during the optimization process.

To all my colleagues in the lab, Lisa Gonçalves, Cláudia Marques, Tomás Azevedo and Nuno Granadeiro, for all the support, mutual aid and companionship.

To Ivette Pacheco, Ana Luísa Santos, Cláudia Tavares, Ana Carolina Araújo, Nídia Cunha, Maurícia Vinhas, Paula Félix, Patrícia Dias, André Mozes, João Furtado and Nuno Amaral for all the good times shared during the thesis, helping overcome the daily stress.

To my Salsa Teacher Beatriz Alberteli and my friends Francesca Negro and José Carlos Pinto, for reminding me that there are other pleasures in life parallel with research, but supporting me always in what was more important to me.

To Doctor Alexandra Leonardo and Doctor Olívia Robusto for helping maintain a personal conscience and healthy intellectual structure for performing well my work.

To Professor Catarina Dias, Professor Teresa Chaveca, Professor Heloísa Santos and Professor Carolino Monteiro for their strength and encouragement in accomplishing this thesis that means so much to me.

To my friend Raquel Cabaços for being always there in the good and bad moments, despite the physical distance that separates us.

To Professor José Bragança for his permanent knowledge, support, confidence, patience and above all his friendship.

To my father for always being there, helping with the papers and treatment of the embryo images.

I would like to say thanks to all my family for their support, concern and help. It would be pretty hard without them.

II. ABSTRACT

Somitogenesis is a crucial process in vertebrate embryo development, whereby mesodermal-derived structures, called somites, are repeatedly formed along the anterior-posterior (AP) body axis. A molecular clock, first evidenced by cyclic expression of the *hairyl* gene, sets the pace of somite formation. Although somites are morphologically identical, they are actually molecularly different. The identity of each embryonic segment is determined by combinatorial expression of *hox* genes. Previous studies showed that *Hairy1* protein interacts with the mesodermal identity protein *Brachyury*, which is also required for *hox* gene activation.

To assess if the embryonic clock is timing *hox* gene activation, *hairyl* and *brachyury* were cloned into an expression vector containing a fluorescent reporter gene. The resulting plasmids were subsequently electroporated in chick embryos at stage 3HH-4HH and the embryos incubated for further development. Phenotype analysis of electroporated embryos suggests that over-expression of either *hairyl* or *Brachyury* genes delays or even prevents somite formation, to approximately the same extent. Furthermore, when the C-terminal domain of *Hairy1*, capable of interacting with *Brachyury*, was over-expressed, a dose-dependent phenotype was obtained, suggesting that it resulted from *Brachyury* protein titration. These results strongly suggest that cyclic *Hairy1* - *Brachyury* dimer formation is required for somite segmentation.

Keywords: *Hairy1*, *Brachyury*, electroporation, molecular clock.

III. SUMÁRIO

No desenvolvimento embrionário existem várias fases importantes, sendo a somitogénese uma delas. Nesta etapa, o embrião forma periodicamente estruturas metaméricas designadas sómitos que se vão posicionando aos pares ao longo do eixo anterior-posterior (AP) do embrião, flanqueando o tubo neural. Um relógio molecular, primeiramente descrito pela expressão cíclica do gene *hairy1*, vai marcando o ritmo da formação dos sómitos. Apesar de parecerem morfologicamente idênticos, os sómitos são molecularmente bem diferentes. Esta identidade espacial ao longo do eixo AP do embrião é dada pela expressão conjunta de diferentes genes *Hox*. Estudos prévios demonstraram que a proteína Hairy1 interage com o marcador de mesoderme Brachyury, que é essencial para a expressão dos gene *Hox*.

Para esclarecer se o relógio embrionário controla temporalmente a expressão dos genes *Hox*, *hairy1* e *brachyury* foram sobre-expressos em embriões de galinha do estadio 3-4 HH usando a técnica de electroporação. A análise morfológica destes embriões sugere que a sobre-expressão de *hairy1* ou de *brachyury* atrasa ou até previne a formação de sómitos, de forma semelhante. Aquando da sobre-expressão da porção C-terminal de Hairy1, capaz de interagir com Brachyury, observou-se um fenótipo intermédio e dependente da dose, o que sugere um efeito de titulação de Brachyury. No conjunto, os resultados obtidos suportam a hipótese de que o dímero Hairy1-Brachyury é necessário para a segmentação somítica.

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1. INTRODUCTION

1.1. General considerations on Development Biology

For centuries Man has been interested in the study of the process by which living beings grow and develop - Developmental Biology – especially from fertilization until birth - Embryogenesis. A wider knowledge of the embryonic development may allow us to intervene in medically assisted reproduction, to avoid or to prevent some malformations, scan diagnoses and apply multiple therapeutic solutions.

Developmental biology studies employ the use of animal models which allow the use of their embryos without raising the ethical questions underlying the use of human embryos. Among the most utilized animals are the invertebrates: Sea urchin (*Strongylocentrotus purpuratus*), Roundworm (*Caenorhabditis elegans*), Fruit fly (*Drosophila melanogaster*); and the vertebrates: Zebrafish (*Danio rerio*), Frog (*Xenopus laevis*, *Xenopus tropicalis*), Mouse (*Mus musculus*) and Chicken (*Gallus gallus*). Their comparison is possible because, in the early stages of development, all embryos present comparable morphology, and the physiological and genetic processes are conserved between species.

The chicken as a model has important advantages, including having a rapid development (21 days total), being accessible throughout the year and in the desired amount, it can be incubated to develop when it is convenient, it is sufficiently transparent and the embryo is sufficiently large to be observed, embryonic development allows external manipulation of the embryo itself by simply opening the egg shell and re-closing it to allow further post-manipulation development, it possesses an embryonic development similar to the Human, both at cellular and molecular level, it is cheap and its development is well described and classified in various stages (the most widely used is Hamburger & Hamilton (HH) (Hamburger and Hamilton, 1951 – Appendix 1).

1.2. Chick Embryogenesis

Embryogenesis begins with the **fertilization** of the egg by sperm to create a new individual with genetic material derived from both progenitors. The zygote suffers a cytoplasmic arrangement with rapid mitotic divisions producing numerous smaller cells, called blastomeres. This process is called **cleavage** and leads to the development of a

multicellular organism - morula. At the end of this stage, that ball of cells becomes hollow, with a single layer of cells – blastoderm – surrounding the internal cavity – blastocoel – and it is now called blastula. Depending on the amount and distribution of yolk in the egg, the cleavage can be holoblastic (the cleavage extends in the entire egg – mammals, amphibians, nematodes) or meroblastic (the cleavage is only in a portion of the egg – fish, reptiles, birds, insects) (Gilbert, 2006).

In the chick, cleavage occurs only in the blastodisc, a small disc of cytoplasm 2-3 mm in diameter at the top of a large yolk (Gilbert, 2006). The blastoderm is initially a unique layer of cells. After this, it is cleaved into a tissue five to six cell layers thick. Between the blastoderm and the yolk is a space called the subgerminal cavity (Gilbert, 2006). The cells in the center of the blastoderm die, remaining only one layer of cells: area pellucida (Fig. 1-1), that is the most important part of the embryo. Surrounding the referred layer, there is a group of cells in the form of a ring that have not lost their deep cells form the area opaca (yellow layer in Fig.1-1) (Gilbert, 2006). Very close to the acellular vitelline membrane that surrounds the yolk (“dorsal” side), is a simple, one-cell-thick epithelium that is continuous throughout area opaca and area pellucida – epiblast (grey layer in Fig. 1-1) (Stern *et al.*, 2004). Other area pellucida cells have delaminated and moved individually into the subgerminal cavity, thus forming the polyinvagination islands (primary hypoblast), a group of islands of separated clusters that hold 5 to 20 cells each (Fig. 1-1 and Fig. 1-2) (Gilbert, 2006).

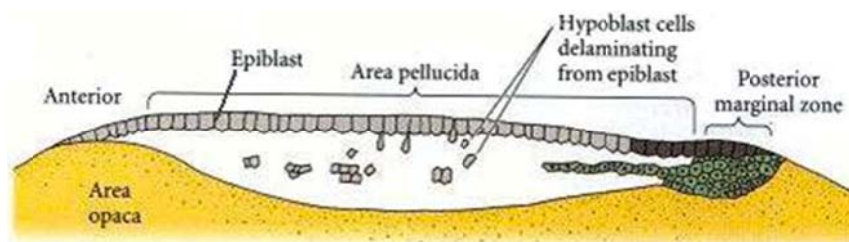


Fig. 1-1 – Lateral view of an embryo forming the hypoblast layer from the epiblast (adapted from Gilbert, 2006).

Between the area opaca and the area pellucida a layer called marginal zone (see Fig. 1-1) is formed, which is very important to determine the cells' fate and to establish the anterior-posterior (AP) axis of the embryo. The boundary between the area pellucida and the marginal zone is marked by a crescent-shaped ridge of small cells, tightly adherent to the epiblast – Koller's sickle (red cells in Fig1-2). Together, these components define a blastoderm of stage

X (Roman numerals from I to XIV are used to classify stages before formation of the primitive streak according to Eyal-Giladi and Kochav – 1976) that represents the embryo when the egg is laid (Stern *et al.*, 2004).

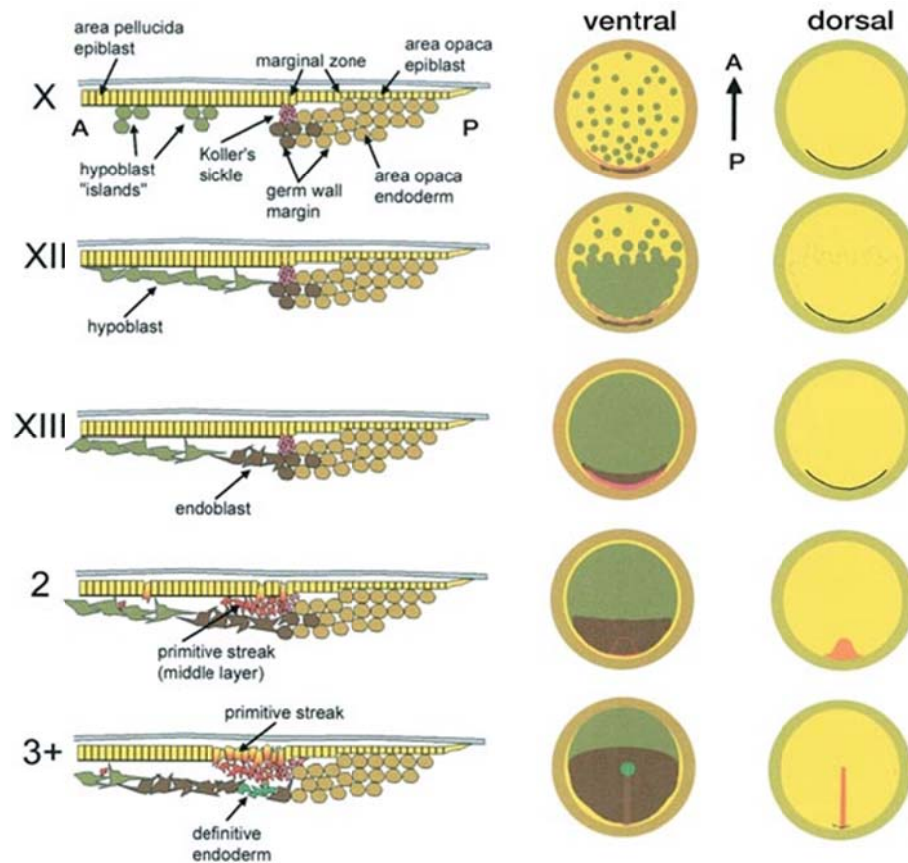


Figure 1-2 - Representative scheme of primitive streak formation. Representative scheme of chicken embryo development since stage X until stage 3⁺HH, seen from 3 different perspectives. The formation of the primitive streak is shown in detail, where A represents the anterior part of the embryo and P the posterior part of the embryo (from Stern, 2004).

The embryonic disk becomes oval in a process called **gastrulation**, when cellular movements give rise to new positions and new neighbors, and the multilayered body plan of the organism is established. The islands of hypoblast gradually fuse together from posterior to anterior to generate a continuous but relatively loose layer, the hypoblast proper (green layer at st. XII in Fig. 1-2). This layer covers half of the area pellucida at stage XII and almost all of it at stage XIII (Stern *et al.*, 2004). Shortly after, the hypoblast is displaced anteriorly and forms the endoblast (or secondary hypoblast) (brown layer at st. XIII in Fig. 1-2). The space between the epiblast and hypoblast is called blastocoel. After this, endodermal precursors ingress from the epiblast into the blastocoel and other cells migrate from the lateral region of

the posterior epiblast to the center appearing a posterior thickening (the posterior bridge), apparently derived from Koller's sickle. This transient structure defines stage XIV and the primitive streak starts to form immediately thereafter (Stern *et al.*, 2004). The streak forms as an accumulation of these cells in the posterior pole of the embryo and extends subsequently in anterior direction defining the axis of the embryo. The cells migrate through the embryo's dorsal side and move to the ventral side (Fig. 1-3). After the mid-streak (3HH) stage, a depression forms within the streak - primitive groove - and starts the migration of the mesenchyme of the streak to establish the lateral plate (3⁺HH) with the three layers: endoderm, mesoderm and ectoderm (Stern *et al.*, 2004). Each layer has distinctive characteristics and gives rise to certain tissues of the body: the ectoderm gives rise to epidermis structures, neural crest and neural tissues, which give rise to the nervous system; the mesoderm gives rise to somites, notochord, blood and blood vessels, bone and connective tissue; the endoderm gives rise to epithelium of the digestive system and respiratory system and organs associated with the digestive system, such as the liver and pancreas (Gilbert, 2006).

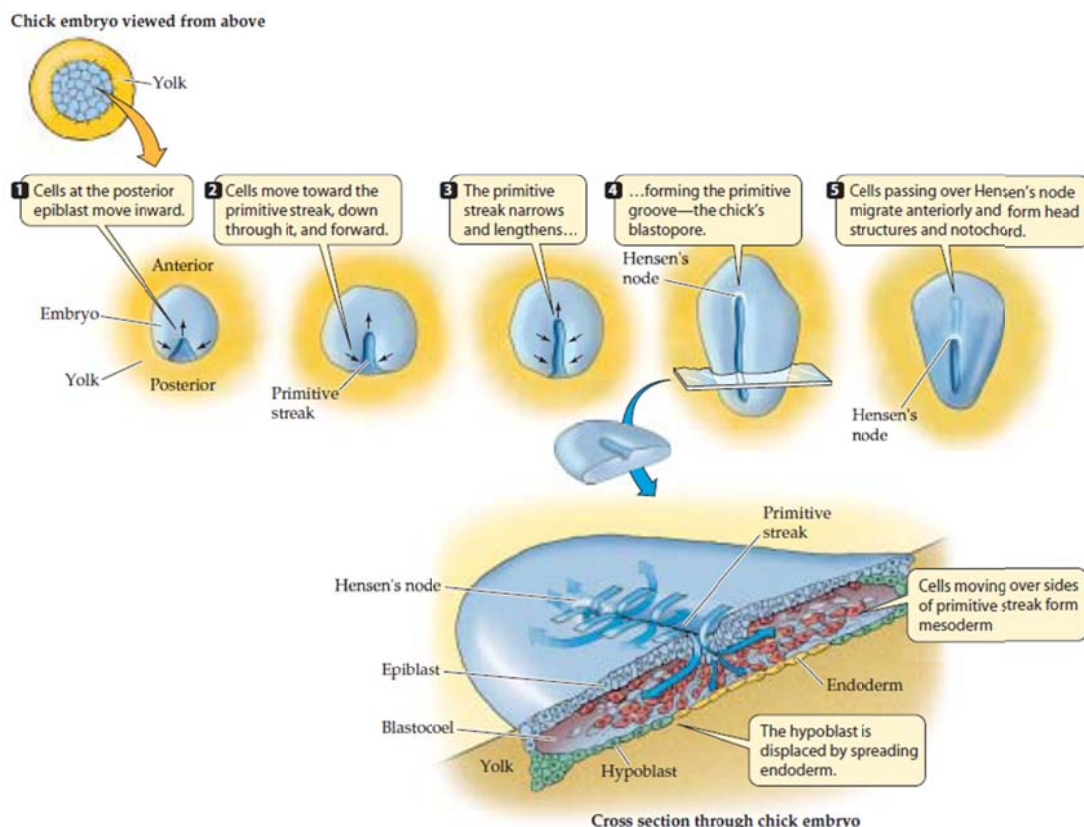


Figure 1-3 - Schematic diagram of the Gastrulation in birds. Gastrulation in chicken embryo with cross-section (stage 4HH) showing the cellular movements when the three germ layers are formed (adapted from Purves *et al.*, 2004).

The anterior end of the streak broadens to form the Hensen's node, a structure roughly equivalent to the organizer of xenopus embryos and the shield of zebrafish, and defines the 4HH with full extension of primitive streak. This is the last phase of the “gastrula stage” in avian embryos (Stern *et al.*, 2004). Along the primitive streak in this stage, the cells' fate varies depending on their position along the AP axis. That fate map of the epiblast cells along the AP axis lateral to the primitive streak was described by Stern in 1996, reviewed by Solnica-Krezel in 2005 and more carefully characterized by Iimura and Pourquié, in 2006. The anterior-most epiblast that lies adjacent to the node and the anterior primitive streak (90% level) essentially gives rise to neural and ectodermal derivatives. More posteriorly, extending from the 90–60% level of the primitive streak, a territory essentially contained paraxial mesoderm precursors is found. Posterior to this territory, the epiblast contributes to the lateral plate and extraembryonic mesoderm (Iimura and Pourquié, 2006).

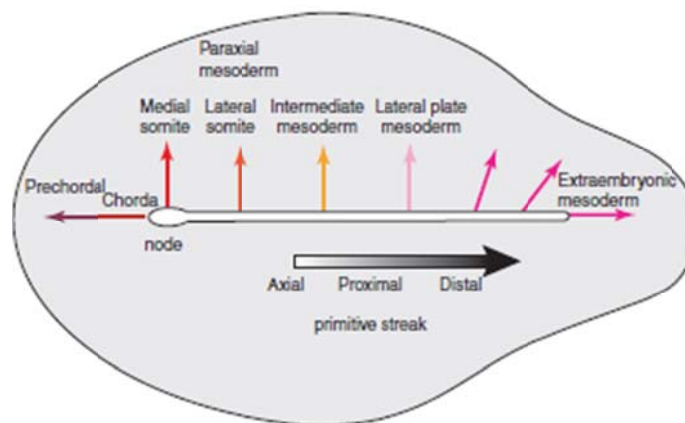


Fig. 1-4 – Schematic representation of the fate map cells in the primitive streak. Neural cells at the anterior top of the primitive streak, somite precursors at the third anterior of the embryo and extraembryonic tissue at the posterior part (Adapted from Solnica-Krezel 2005).

Shortly afterward, the Hensen's node starts to regress posteriorly and leave a rod of condensed mesoderm anteriorly - the notochord or head process (Stern *et al.*, 2004). At the same time, the future neural plate starts to become morphologically and molecularly distinct, with the beginning of the “neurula stage” (5HH). At 6HH, a definite fold of the blastoderm anterior to the notochord marks the anterior end of the embryo. These structures can be seen in different stages in Fig.1-5.

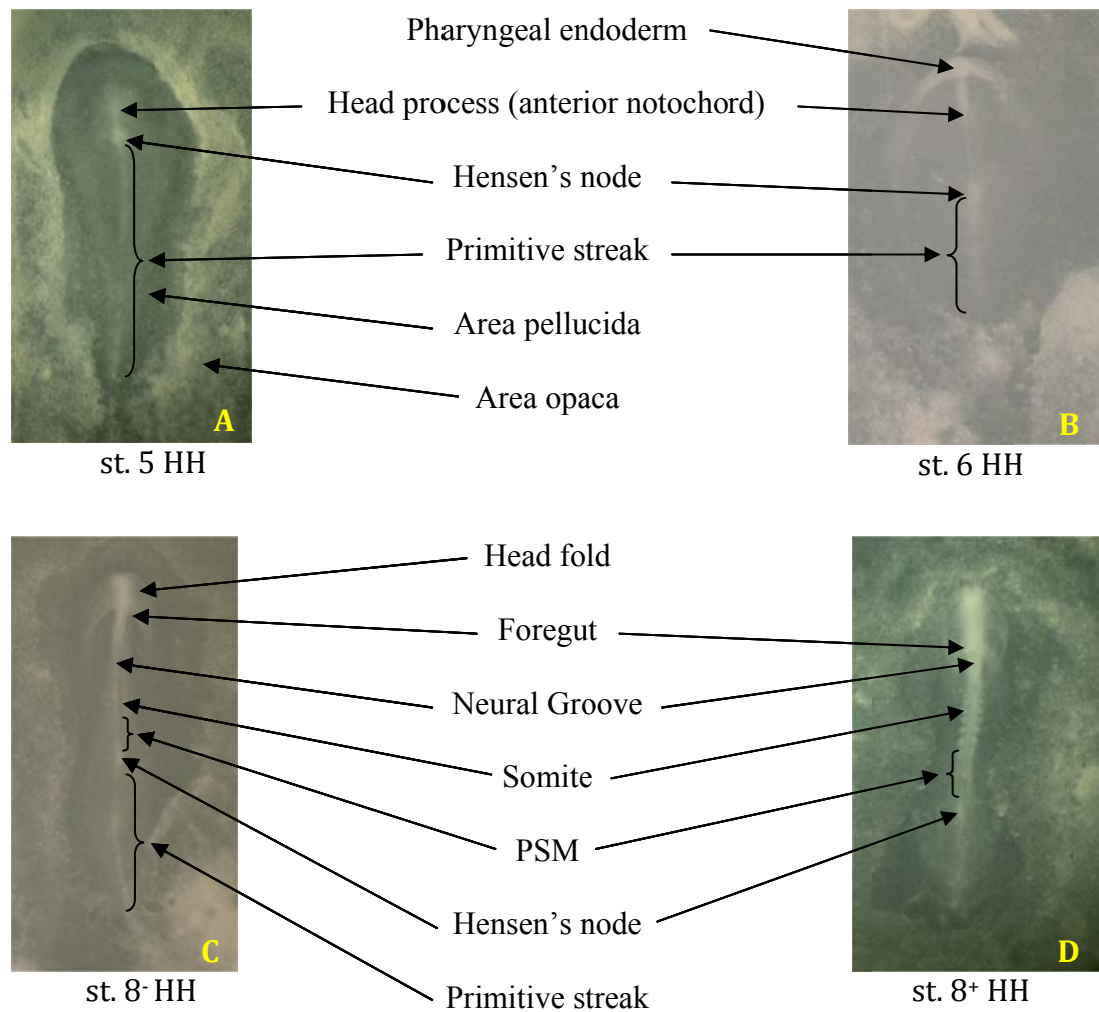


Figure 1-5 Embryos structures at different stages. Illustration of the several morphological features of the chicken's embryos at different stages: A – stage 5HH, B – stage 6HH, C – stage 8HH, D – stage 8⁺.

Neurulation in vertebrates results in the formation of the neural tube from the neural plate, which gives rise to both the spinal cord and the brain. Before long after the neural plate has been formed, its limits get thick and travel upward to form the neural folds, as a neural groove shaped as a U appears in the middle of the plate, in-between the future right and left sides of the embryo (Gilbert, 2006). The neural folds wander in the direction of the midline of the embryo, finally fusing to structure the neural tube underneath the overlying ectoderm. The cells at the dorsal-most portion of the neural tube are the neural crest cells (Gilbert, 2006). During neurulation, somites form in pairs flanking the neural tube, appearing the first pair at 7HH by a process called **somitogenesis**.

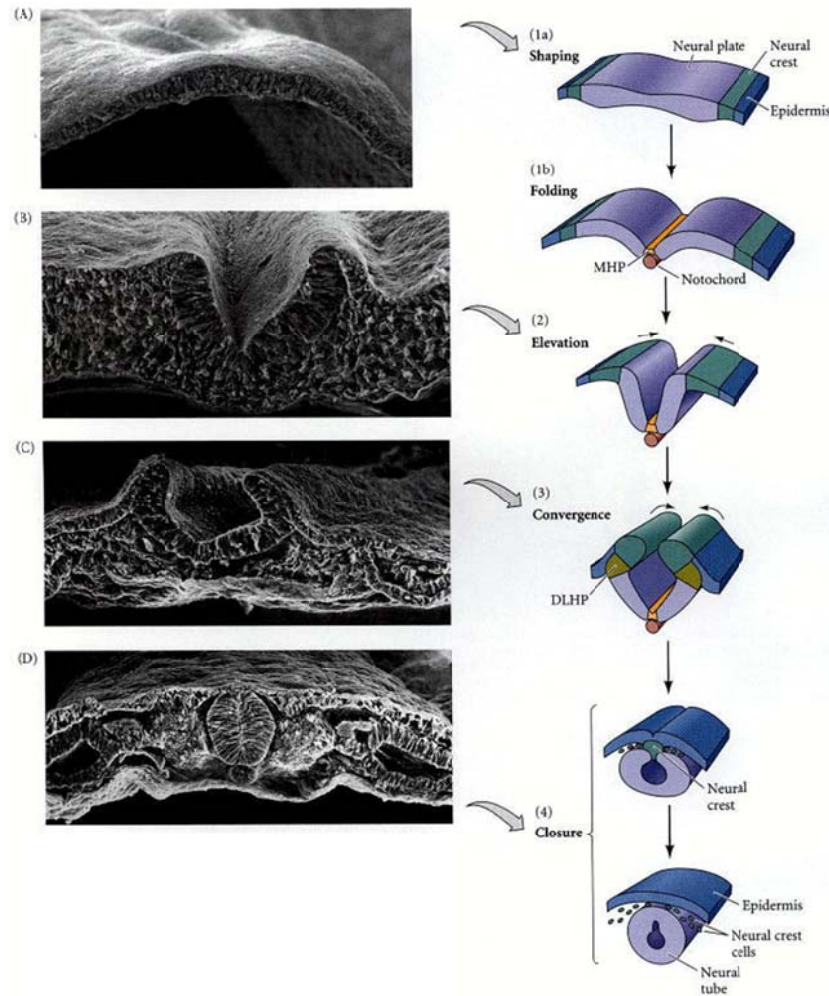


Figure 1-6 - Neurulation Process. Representative schematic of the neurulation process accompanied by scanning electron microscopy images since the neural plate (A) to the formation of the neural tube flanked by the somites (D) (adapted from Gilbert, 2006).

Simultaneously with neurulation, the embryo also folds ventrally to enclose the gut and bring the two heart primordia together to fuse, starting the **organogenesis**.

1.3. The Somitogenesis clock

During development, embryos exhibit a distinct anterior-to-posterior gradient of developmental maturity (Gilbert, 2006). This is particularly evident during the somitogenesis process, wherein the vertebrate embryo body is segmented periodically into repeated units, called somites, in a head-to-tail sequence. In the anterior part of the embryo, formed somites begin to differentiate into various tissues (e.g., vertebrae, ribs, intercostal muscles, dermis of

the back and tendons), whereas at the same time in the posterior part, the presomitic paraxial mesoderm (PSM) remains unsegmented and undifferentiated (Aulehla and Pourquié, 2010). These transitory structures (somites) are a group of cells that suffered a mesenchymal-epithelial transition at the anterior tip of the PSM, and are symmetrically positioned relative to the neural tube and notochord and they represent the first overt sign of a segmented body plan.

If it is true that the morphological difference is only noticeable at the anterior tip of the PSM where cells become epithelialized, at the molecular level, that difference is already observed along the anterior third of the PSM tissue, where somite anterior-posterior (A-P) polarity is specified by *Mesp2* and Delta/Notch signaling (Saga, 2007).

Along the PSM, the cells exhibit different levels of differentiation where the segmental determination takes place in the PSM around the level of somite –IV, that correspond to the **determination front**. Here, Dubrulle *et al.*, (2001) showed that fibroblast growth factor 8 (*fgf8*) was expressed as a gradient from the posterior part, decreasing towards the anterior PSM, by the progressive decay of *fgf8* mRNA generated at the tail bud of the embryo, defining what was termed a wavefront of differentiation (Dubrulle and Pourquié, 2004a). Additionally, when *fgf8* was over-expressed in the undetermined caudal region of the PSM the position of somitic boundaries had changed creating small somites and an activation of ectopic expression of genes like *hoxB9* or *hoxA10* was observed in order to maintain the anterior limit of expression of these *Hox* genes at the appropriate somitic level (Dubrulle *et al.*, 2001).

Concomitant with the *fgf8*'s gradient, mouse embryos also present a Wnt signaling gradient (Aulehla *et al.*, 2003), as *Wnt3a* expression is increased in the most posterior PSM and tail bud. A reversely oriented gradient of retinoic acid (RA) is also established, presenting higher expression in the somites and anterior PSM, whereas it is absent in more posterior parts of the embryo, including the tail bud (Diez del Corral *et al.*, 2003). This combinatorial gradient system in the PSM that involves the activity of FGF, Wnt and RA signaling pathways is a fundamental key to maintain the undifferentiated state of cells in the posterior PSM and to control initiation of differentiation once cells reach the anterior PSM (Fig. 1-7). High levels of Wnt and FGF signaling maintain cells in an undifferentiated, posterior PSM state, whereas the exposure to high levels of RA signaling (and concomitant low levels of Wnt and FGF signaling) is responsible for initiating differentiation (Aulehla and Pourquié, 2010).

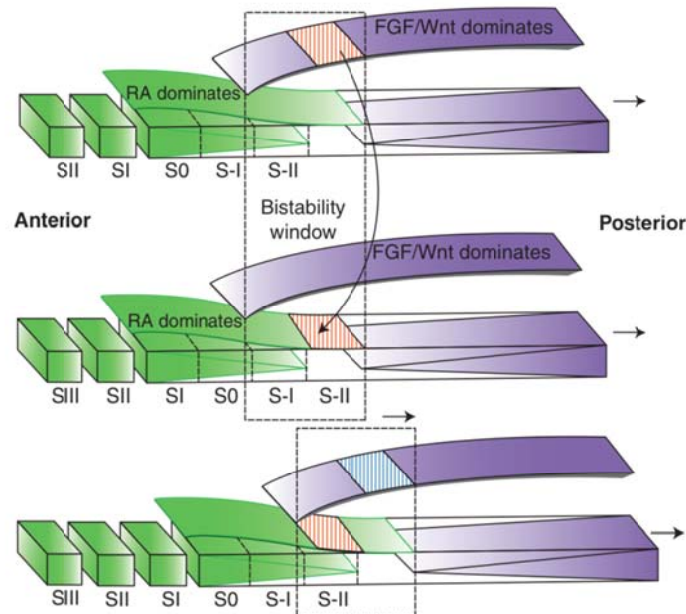


Figure 1-7 - Model for segment determination through opposing gradients. From the relationship of two types of gradients (FGF/Wnt in the posterior part and RA in the anterior part) a bistability window is established, where probably the segmentation clock acts to define the transition of the more undifferentiated cells to a cohort of cells progressively more differentiated and organized (orange). This window moves posteriorly over time, appearing new cells in the place of the first ones (blue) (from Aulehla and Pourquié 2010).

A very important piece of the puzzle was discovered in 1997 by Palmeirim *et al.* The **somitogenesis molecular clock** was firstly evidenced by cyclic *hairy1* mRNA expression in the chick PSM, appearing as a caudo-rostral wave, reiterated during the formation of every somite (Fig. 1-8). Coincidentally, the time that is needed to perform a cycle of gene expression corresponds to the formation time of one somite (90 min) (Palmeirim *et al.*, 1997). So, a molecular clock times vertebrate embryo somite formation. This periodic mechanism is repeated multiple times until the embryo acquires a defined species-specific final number of somites at the end of the process of axis elongation and presents a periodicity which is also species-specific (Tenin *et al.*, 2010). In the chick embryo, a somite pair is laid down every 90 min in a rostro-caudal progression (Palmeirim *et al.*, 1997), and a total of 51-53 somite pairs are formed during embryogenesis (Tenin *et al.*, 2010).

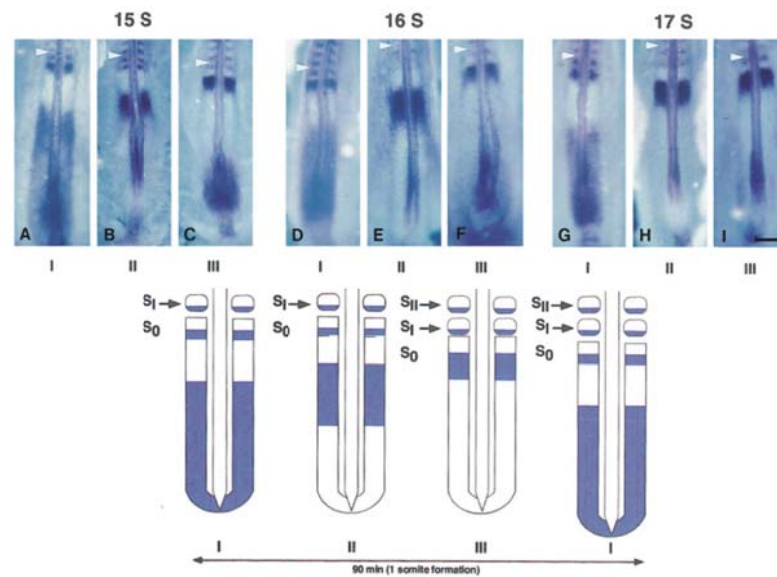


Figure 1-8 - *Hairy1* mRNA expression pattern. The dynamic *Hairy1* expression over 90 min (that is the same time to form a new somite) observed by *in situ* hybridization was considered the first gene from a molecular clock that regulates the segmentation (Palmeirim *et al.*, 1997).

Thereafter, signaling components, including many negative feedback inhibitors of the Notch, Wnt, and Fgf pathways, are involved in the segmentation clock and have been proposed to control the periodic activation of the system (Iimura *et al.*, 2009). Null mutations involved in the Notch signaling pathway or downstream targets, like *lfng*, *hes1*, *hes5*, *hes7* or *dll1* produce segmentation defects, confirming Notch signaling as an important component of the segmentation clock (Andrade *et al.*, 2007; Duncan *et al.*, 2007). FGF and Wnt pathways play an important role too. *nkd1* and LEF/TCF expression belonging to the Wnt signaling are two examples of the reciprocal interaction of Notch and Wnt signaling pathways in the somitogenesis clock (Andrade *et al.*, 2007). From this three signaling pathways, the FGF and Notch clusters of cycling genes are activated in parallel, while genes belonging to the Wnt cluster present an opposite phase of expression (Dequéant *et al.*, 2006).

1.4. *Hox* genes in embryonic development

Besides the important function of controlling cellular differentiation and the formation of segmental unit, the gradient system of Wnt, FGF and RA signaling is also involved in the control of *Hox* gene expression in the PSM (Iimura *et al.*, 2009). Although initially somites all look morphologically alike, they will subsequently develop into very different structures

with unique functions depending on their axial position – cervical, thoracic, lumbar and sacral vertebrae, for example (Aulehla and Pourquié, 2010). This axial identity is given by the action of **Hox** genes, which encode homeobox-containing transcription factors.

An ancestral *Hox* gene cluster is proposed to have been repeatedly duplicated, resulting in four sets in birds and mammals (Iimura and Pourquié, 2007). In each cluster, the 3' *Hox* genes are expressed first, whereas more 5' *Hox* genes are expressed later and sequentially (Fig. 1-9). This phenomenon has been called “**temporal collinearity**” (Deschamps and Van Nes, 2005). Moreover, the *Hox* genes are arranged on the chromosome in a sequence that reflects not only their order of expression initiation during embryogenesis, but also the anterior limit of their expression domains along the A-P axis, thus defining what has been called as “**spacial collinearity**” (Iimura and Pourquié, 2007). From the distribution of the different *Hox* genes along the embryo, result the different AP identities of the vertebrae.

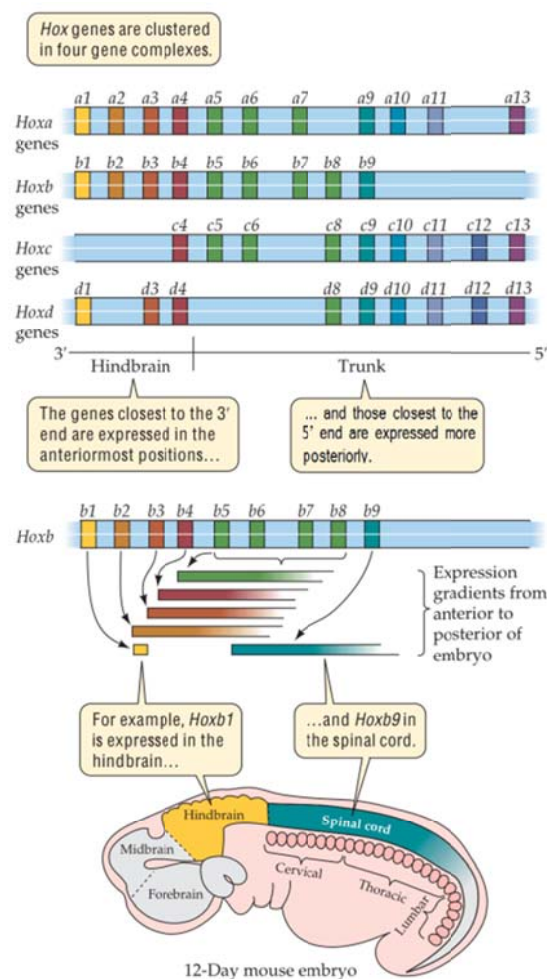


Figure 1-9 - Distribution of the *Hox* genes. Schematic representation of the *Hox* genes distribution along the 4 chromosomes and in the anterior-posterior axis of mouse's embryo, showing a spatial collinearity (adapted from Purves *et al.*, 2004).

Hox gene expression initially appears in early stages of chick embryo development, as a salt-and-pepper pattern at the posterior primitive-streak level and progressively extends anteriorly in the epiblast along the primitive streak. Then, expression of *Hox* genes progressively spreads to the surrounding cells, resulting in their expression in virtually all of the epiblast cells along the streak up to the node level. Next, *Hox* gene expression subsequently spreads even further anteriorly to the node in the posterior neural plate (Fig. 1-10) (Iimura *et al.*, 2009). These expression dynamics follow the gastrulation movements of cells from the epiblast to the mesoderm, suggesting that *Hox* genes could play a role in mesoderm formation through gastrulation (Iimura and Pourquié, 2007).

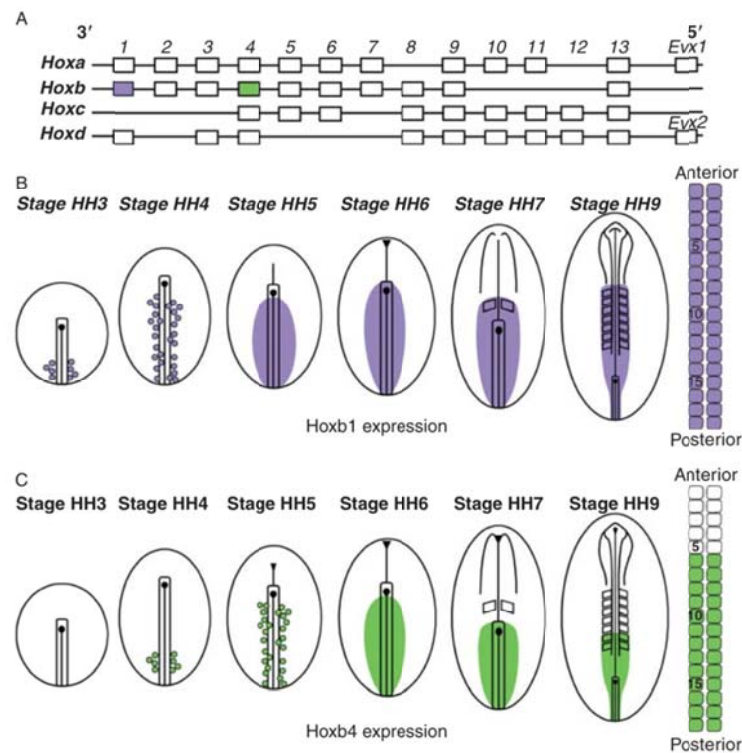


Figure 1-10 - *Hox* gene activation in the chicken embryo. *Hox* gene distribution on four chromosome clusters, showing spatial collinearity (A); Activation of *hoxb1* along chicken embryo development (B); *hoxb4* expression pattern along the chicken embryo, showing a posterior activation to the *hoxb1*, agreeing with the spatial collinearity of the *Hox* genes (C) (from Iimura *et al.*, 2009)

Activation of the *Hox* genes is initiated in the epiblast in a collinear fashion before their ingress into the primitive streak (Iimura and Pourquié, 2006). The *Hox* genes are thus all activated in a similar fashion, following a temporal collinear sequence. However, the identity of each segment throughout the AP axis is controlled only by the posteriormost *Hox*

gene that is expressed in that segment. This property is termed **posterior prevalence** (Duboule and Morata, 1994).

The molecular mechanism driving the sequential activation of *Hox* genes in the epiblast is unknown. In vertebrates, it has long been believed and recently shown that temporal collinearity reflects the **progressive opening of chromatin** in a 3'-to-5' direction, thus providing temporally controlled access of the transcription machinery to *Hox* gene promoters (Soshnikova and Duboule, 2009). The collinearity has also been proposed to reflect the action of signaling pathways on **cis-regulatory elements** dispersed in the *Hox* clusters during gastrulation. The timing of activation of a *Hox* gene in the cluster depends on its position within the cluster, on local *cis*-regulatory sequences, on chromatin remodeling machinery and specific regulatory elements located outside of the cluster (Iimura *et al.*, 2009).

Initial activation of *Hox* gene transcription and the early anterior propagation event of *Hox* gene expression could be regulated by molecular events involved in gastrulation, including the formation and caudal regression of the primitive streak (Pourquié, 2007). **Fgf** signaling is essential for mesoderm formation through the primitive streak and its caudal regression, suggesting that it could play a regulatory role in the initial expression of *Hox* genes (Dubrulle *et al.*, 2001). It has also been proposed that **RA** could play a role in vertebral patterning, in part, via regulation of *Hox* expression because RA treatment leads to distinct homeotic transformations and acts differentially on 3' vs 5' *Hox* genes in mesoderm (Kessel and Gruss, 1991). **Wnt** signaling has also been connected directly to the anterior progression of *Hox* genes since Wnt regulates the formation of the primitive streak (Ikeya and Takada, 2001; Aulehla and Pourquié, 2010). Finally, the caudal transcription factors (**Cdx**) are targets of the Wnt, FGF, and RA pathways, which play a conserved role in *Hox* regulation during AP axis formation (Iimura *et al.*, 2009). The correlation of these pathways is schematically represented in Fig. 1-11.

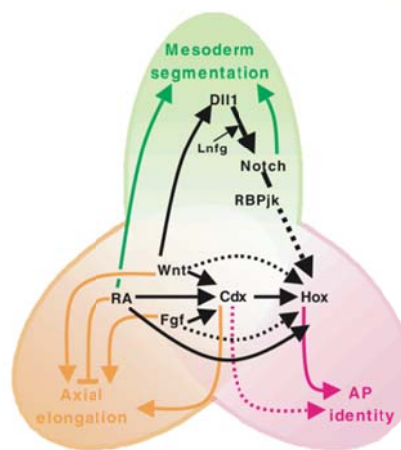


Figure 1-11 - Connection between the *Hox* genes and multiple signalling pathways. FGF, Wnt and RA signalling are involved in axial extension (orange), somitogenesis (green) and AP patterning (purple). *Hox* genes are regulated by these pathways, along with Notch signalling and *Cdx* genes. Unbroken lines indicate established interactions; broken lines represent documented interactions that have not yet been established at a molecular level (from Deschamps and Van Nes, 2005).

Still, a number of experiments suggest that the clock and wavefront mechanism that drives the periodic segmentation of PSM tissue into somites also regulates *Hox gene* expression (Krumlauf, 2009). The spatial regulation of *hoxd* genes in the anterior PSM is controlled by the segmentation machinery with the influence of the **Notch** pathway (Zakany *et al.* 2001). Besides that, loss- or gain-of-function mutations in genes of the Notch pathway affect *Hox* gene expression in the PSM and subsequently, skeletal patterning (Cordes *et al.* 2004).

1.5. Brachyury

"It is not birth, marriage, or death, but gastrulation, which is truly the most important time in your life"(Lewis Wolpert, 1986). During gastrulation, the formation of a mesodermal layer of cells between the endoderm and the ectoderm is a fundamental step. One of the earliest mesoderm markers is *brachyury* (*T*). Its expression is restricted to the nascent mesoderm, with an essential function for the migration of mesodermal cells through the primitive streak, and later in the tail bud (Wilson and Beddington, 1997). *brachyury* is the founding member of the T-box family of transcription factors, which is characterized by the N-terminal conserved DNA-binding T-domain.

In *Xenopus laevis* embryo, AP positional information for the vertebrate trunk has been proposed to be generated by sequential interactions between a timer in the early non-organizer mesoderm and the Spemann organizer (Durstun *et al.*, 2009). This results in temporal collinear activation of *Hox* genes in the early ventral and lateral mesoderm (i.e., the non-organizer mesoderm) of the *Xenopus* gastrula. In this system, the mesodermal transcription factor *brachyury* (*Xbra*) is required for early *Hox* expression domain in the animal–vegetal direction and the secreted growth factor *BMP-4* limits it in the organizer/non-organizer direction (Wacker *et al.*, 2004a). The overlap of these two signals defines the initial *Hox* expression domain. Therefore, the contribution of T in *Hox* induction, and as well as the requisite for a molecular timer in the mesoderm have both been proposed to control collinear *Hox* gene expression activation.

1.6. Manipulating gene expression levels in the chick embryo:

Electroporation and Explant culture

The basic principle of electroporation is that application of an electric field to cells generates the reversible opening of pores in the cellular membrane, which then allows charged macromolecules, such as DNA, RNA, and proteins, to penetrate the cells (Iimura and Pourquié, 2008). Muramatsu *et al.* (1997) first reported the use of such electroporation techniques *in ovo* to over-express genes in the chick embryo. Subsequently, the conditions for routine use of *in ovo* electroporation in various tissues have been established, and a wide range of potential applications has been developed (Iimura and Pourquié, 2008). Nevertheless, it has been difficult to establish electroporation methods for the epiblast in early gastrulating embryos (stages 3 to 7HH) because of their small size and fragility. The use of flat electrodes, combined with an *in vitro* culture system, has made it possible to overcome these issues and over-express genes in embryos at the primitive streak stage (Iimura and Pourquié, 2008).

From the several methods available for explants culture upon electroporation, two are currently widely used: the ‘EC culture’ method (Chapman *et al.*, 2001), in which the embryo attached to its own vitelline membrane is explanted by attaching the outer portion of the membrane to a piece of filter paper and subsequently transferred to an agar substrate on which it is cultured, and New culture (New 1995), involving removal of the embryo attached to its

own vitelline membrane, which is then stretched around a glass ring and cultured on a pool of egg albumen (Voiculescu *et al.*, 2008). The EC culture has several benefits compared with classical New culture: (1) It does not require either glass rings or watch glasses; (2) standard laboratory filter paper is inexpensive and readily available, and it can be sterilized by autoclaving; (3) Tension on the blastoderm is maintained without the need to adjust the vitelline membranes on a glass ring placing the filter paper directly onto the intact yolk before cutting through the vitelline membranes; (4) Cultures can be made very quickly; (5) Identical conditions are maintained for each embryo; (6) Liquid does not pool on the embryo; (7) Embryos can be easily removed and replaced on the substratum and, therefore, can be cultured first ventral-side up and then dorsal-side up (after they reach stage 8HH); (8) Embryos typically develop at about the same rate as *in ovo*, and development of the extraembryonic vasculature is extensive; (9) Embryos can be routinely cultured until they reach stages 15–17, although expansion of the rostral brain region is retarded in such embryos, and sections reveal that some degeneration of the neural tube occurs (Chapman *et al.* 2001).

1.7. Aims of this study

This study intended to contribute to the clarification of a fundamental developmental question regarding how temporal control of positional information is achieved. If, on one hand the molecular clock is giving a notion of time by the number of expression cycles of certain genes that the cells received, on the other hand the sequential expression of *Hox* genes confers spatial identity to the PSM cells. Additionally, Hairyl protein interacts with Brachyury (unpublished results) and our available data suggest that the protein dimer Brachyury-Hairyl could be involved in *Hox* gene transcription regulation, thus implicating the molecular clock in both temporal and spatial control of cell identity.

To test this hypothesis, plasmids were constructed to over-express *hairyl* and *brachyury in vivo*, employing the embryo culture system (Chapman *et al.* 2001) to re-incubate the embryos after electroporation for an appropriate period of time. The empty vector was used as control. Successful electroporation was confirmed by *in situ* hybridization with probes to label cells that are actively transcribing each gene. Afterwards, a careful analysis of the

morphological characteristics of the embryos was performed with the aim of finding correlations between the phenotypes obtained and the different expression plasmids. Perturbing the system with either *hairyl* or *brachyury* over-expression could result in common external embryo alterations if they are working in the same way. Electroporated embryos were also collected for posterior analysis of *Hox* gene expression by reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) assays.

2. MATERIALS AND METHODS

2.1. pCAT

2.1.1. Plasmids construction

pCAT was generated by cloning the IRES-MCS region from pIRES2-GFP (Clontech) into pCAGGS-AFP (kindly provided by Tsuyoshi Momose). This fragment was generated by PCR using the MCS-IRES-ForSpeI (GGACTAGTGCTAGCGCTACCGGACTCAG) sense primer and the antisense primer MCS-IRES-RevSpeI (GGACTAGTTGTGGCCATATTATCATCGT). To confirm the length of the fragment, an agarose gel electrophoresis was performed. The bands were cut and purified by QIAquick® Gel Extraction Kit (Qiagen). The products were digested with the restriction enzyme *SpeI* (Fermentas) and the digestion product was checked on a 0.8% agarose gel.

The pCAGGS-AFP vector was linearized with the restriction enzyme *XbaI* (Fermentas) that produces compatible ends with *SpeI*. This product was incubated with Shrimp Alkaline Phosphatase (SAP) (Roche) that catalyzes the dephosphorylation of 5' phosphates from DNA preventing the vector's re-ligation. To confirm completion of the digestion, an agarose gel electrophoresis was performed. Digested products were gel purified using QIAquick® Gel Extraction Kit (Qiagen) and checked on a 0.8% agarose gel to confirm the efficiency of extraction.

The ligation of the two generated DNA products was performed with the Rapid DNA Ligation Kit (Fermentas) using the One Shot® Mach1-T1 Chemically Competent *E. coli* cells (Invitrogen). Eight colonies were selected and grown for plasmid extraction using the QIAprep® Spin MiniPrep Kit (Qiagen). Ligation confirmation and analysis of the fragment orientation was performed by restriction digestion using 2 different enzymes: *EcoRI* (Fermentas) and *BamHI* (Roche). Finally, the selected plasmids were sequenced using the sense primer SeqT3_For (GAGGGCCTTCGTGCGTCG) and antisense primer MCS-IRES-RevSpeI. Plasmid concentration was measured using NanoDrop 2000C Spectrophotometer (Thermo Scientific).

2.1.2. pCAT + c-Term

A C-terminal fragment of the *hairy1* gene (c-Term) was generated by PCR using the sense cTerm-AFP-NheI (CTAGCTAGCCAGATCGTGGCCATGAACTACCTGC) and

antisense Hairy1-AFP-EcoRI (CCGGAATTCCTACCACGGCCGCCAGACG) primers. To confirm the length of the fragment, agarose gel electrophoresis was performed. The bands were excised and purified by QIAquick® Gel Extraction Kit (Qiagen). The products were simultaneously digested with the restriction enzymes *EcoRI* and *NheI* (Fermentas) which were then heat-inactivated. The digestion product was checked on a 0.8% agarose gel.

The previously constructed pCAT plasmid was digested with the MCS restriction enzymes *EcoRI* and *NheI*. This product was incubated with SAP to ensure plasmid dephosphorylation. To confirm the length of the fragment an agarose gel electrophoresis was performed. Digested products were gel purified with QIAquick® Gel Extraction Kit (Qiagen) and checked on a 0.8% agarose gel to confirm the efficiency of extraction.

The ligation of the two fragments was performed with Rapid DNA Ligation Kit (Fermentas) using the One Shot® Mach1-T1 Chemically Competent *E. coli* cells (Invitrogen). 24 grown colonies were expanded and a colony-PCR reaction was performed with the primers cTerm-AFP-NheI and Hairy1-AFP-EcoRI to check for successful clones. These were grown and the plasmid DNA was extracted using the QIAprep® Spin MiniPrep Kit (Qiagen) and a new PCR with the same primers was performed, confirming the length of the fragment in a 0.8% agarose gel. Finally, the plasmids were sequenced using the sense primer SeqT3_For and antisense primer MCS-IRES-RevSpeI. Plasmid concentration was measured using the NanoDrop 2000C Spectrophotometer (Thermo Scientific).

2.1.3. pCAT + Hairy1

The *hairy1* open reading frame (ORF) was obtained by *EcoRI* restriction digestion of the pCRII-TOPO+Hairy1 plasmid available in the lab. To confirm the length of the fragment an agarose gel electrophoresis was performed. The band was cut and purified with QIAquick® Gel Extraction Kit (Qiagen). The digestion product was checked on a 0.8% agarose gel to confirm extraction efficiency.

pCAT was linearized with the MCS restriction enzyme *EcoRI* and the digestion was confirmed by agarose gel electrophoresis.

The ligation of the two aforementioned fragments was performed with the Rapid DNA Ligation Kit (Fermentas) using MultiShot™StripWell TOP10 Chemically Competent *E. colicells* (Invitrogen). Plasmid DNA extraction was made from 12 colonies using QIAprep®

Spin MiniPrep Kit (Qiagen). The plasmids were digested with *EcoRI* to confirm *hairy1* ORF presence and the length of the fragment was confirmed by agarose gel electrophoresis. To confirm the correct orientation of the *hairy1* ORF, two different PCR reactions were performed with the following primers pairs: Hairy1-AFP-*EcoRI* (CCGGAATTCTCTACCACGGCCGCCAGACG) and pCATSeq_REV (ACATATAGACAAACGCACAC); Hairy1-AFP-*NheI* (CTAGCTAGCATGCCCCGCCGACACGGGCATG) and pCATSeq_REV. Only the second primer-pair should result in PCR amplification in a plasmid with *hairy1* in the correct orientation. Finally, selected plasmids were sequenced using the sense primer pCATSeq_FOR (ACTTCCTTTGTCCCAAAT) and antisense primer pCATSeq_REV. The plasmid concentration was measured using the NanoDrop 2000C Spectrophotometer (Thermo Scientific).

2.1.4. pCAT + Brachyury

The *brachyury* ORF was amplified by PCR using the sense primer T-AFP-*NheI* (TTCGCTAGCATGGGCTCCCCGGAGGACGC) and the antisense primer T-AFP-*EcoRI* (CCGGAATTCTTACATGGAAGGTGGGGTGATGGG) on the pSK-T-orf plasmid (kindly provided by Susan Mackem). To confirm the length of the fragment, an agarose gel electrophoresis was performed. The bands were cut and purified using the QIAquick® Gel Extraction Kit (Qiagen). The *brachyury* ORF was then cloned into the pCRII-TOPO® plasmid (Invitrogen), according to the manufacturer's protocol. Four white colonies were selected and plasmid DNA extraction using QIAprep® Spin MiniPrep Kit (Qiagen) was made. The plasmids were digested with the restriction enzyme *EcoRI* to confirm the presence of *brachyury* ORF and the length of the fragment was confirmed by agarose gel electrophoresis. The plasmids were sequenced using the commercial primer T3 (AATTAACCCTCACTAAAGGG). The product of the digestion with *EcoRI* of the correct pCRII-TOPO+Brachyury plasmid was cut, purified using QIAquick® Gel Extraction Kit (Qiagen) and was finally checked on a 0.8% agarose gel. pCAT was linearized with the MCS restriction enzyme *EcoRI* and the digestion was confirmed by agarose gel electrophoresis.

The ligation of the two fragments was performed with the Rapid DNA Ligation Kit (Fermentas) using MultiShot™StripWell TOP10 Chemically Competent *E. coli* cells

(Invitrogen). Eight colonies were grown and plasmids extracted using the QIAprep® Spin MiniPrep Kit (Qiagen). To confirm the orientation of the *brachyury* ORF in pCAT, two different PCR reactions were performed using the following primers: T-AFP-EcoRI and pCATSeq_REV; T-AFP-NheI and pCATSeq_REV. Only the second primer-pair should result in PCR amplification in a plasmid with *brachyury* in the correct orientation. Finally, selected plasmids were sequenced using the sense primer pCATSeq_FOR and the antisense pCATSeq_REV primer. Plasmid concentration was measured using the NanoDrop 2000C Spectrophotometer (Thermo Scientific).

2.2. Embryos and staging

Fertilized chicken (*Gallus gallus*) eggs were obtained from commercial sources (Sociedade Agrícola da Quinta da Freiria, SA.) and stored at 16°C for up to 1 week. Embryos were incubated at 38°C in a humidified chamber for the appropriate time, according to Hamburger and Hamilton (1951).

2.3. Embryo explant culture

Embryo explant cultures were performed according to the EC - Early Chick method (Chapman *et al.*, 2001), as further described. Fertilized chick eggs were removed from the incubator and allowed to cool for 15-30 min before cleaning the shell with 70% ethanol and allowing it to dry. The yolk was separated from the egg white (Fig. 2-1 A; B) and was directly deposited onto a 10-cm glass Petri dish (Fig. 2-1C), such that the blastoderm was positioned centered on the yolk, and the vitelline membrane was intact (Fig. 2-1D).

The thick albumine covering the blastoderm was removed using a piece of sterile filter paper (Whatman no. 3) with gentle movements, drawing it away from the center (Fig. 2-1E), because the albumine interferes with the ability of the vitelline membranes to attach to the filter paper.

Another piece of sterile filter paper with a central aperture was placed gently onto the vitelline membranes, such that the embryo was framed (Fig. 2-1F). The paper immediately absorbed liquid from the surface of the vitelline membrane, drawing it tightly onto the paper.

The vitelline membrane was cut around the filter paper with a scissors (Fig. 2-1G). With forceps, the filter paper was gently pulled away from the yolk in an oblique direction (Fig. 2-1H). Holding the filter paper, any attached yolk was gently removed using blunt forceps (Fig. 2-1I).

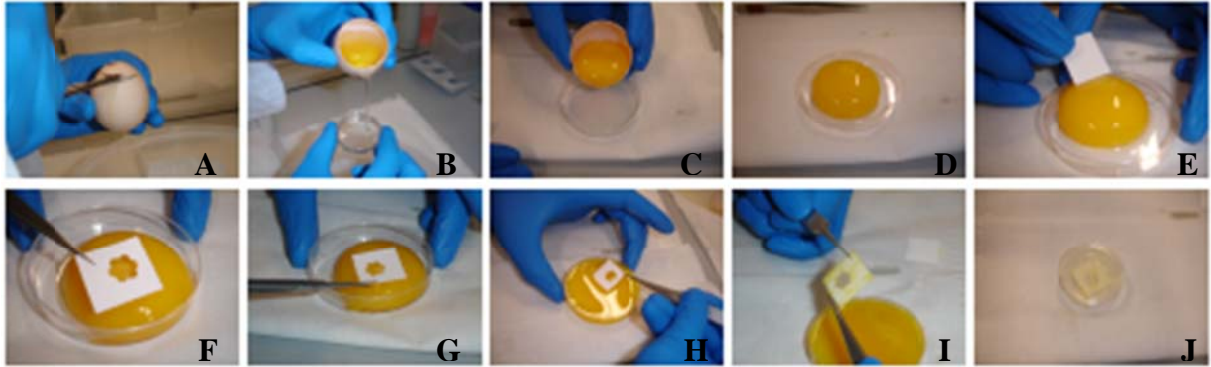


Figure 2-1 - Step sequence of the EC culture execution
Illustrative images showing how to handle the material and the embryo for EC culture (A-J).

The embryo thus prepared was then electroporated as described in section 2.4. After electroporation, the filter paper was placed onto an agar-albumen culture dish with the blastoderm ventral-side up (Fig. 2-1J), carefully to avoid trapping air bubbles between the filter paper and the semi-solid medium. Some thin albumine was placed on the Petri cover to prevent condensation (Fig. 2-1 B). Then the embryo was reincubated at 38°C in a covered 150-mm Petri dish, with moistened tissue paper lining the base, during about 13hours.

2.4. Electroporation of chick embryo tissues

The embryo attached to a filter paper (prepared as described in the previous section) was placed in the inner silicone ring of the CUY700-P2E petri dish-type electrode (NEPA GENE), which was previously fully filled with a Tyrode's saline solution (Stern *et al.*, 2008). All the solutions used in materials and methods are described in Table 2-1. The embryo was positioned over the 2 mm window (ventral side up). A thin glass needle was prepared from glass capillary tubes using a puller (SUTTER INSTRUMENT CO., Model P-87, Flaming/Brown Micropipette) set as follows: Time 150, Heat 720, Pull 75, Vel 60. Plasmid DNA containing 1/5 volume of 0.4% (wt/vol) Fast Green solution was injected into a slit

between the embryo vitelline membrane and the epiblast, targeted lateral to the anterior primitive streak groove. Subsequently, the anode electrode (CUY700-P2L, NEPA GENE) was placed onto the surface of the solution filling the inner ring, ensuring that the electrode covered the targeted area of the embryo (Fig.2-2). In this way, the distance between the two electrodes was always maintained at approximately 4 mm. Five 50 ms pulses of 10 V, with a interval of 350 ms were applied by the electroporator BTX ECM 830, Electro Square Porator (Harvard Apparatus), using a foot pedal.

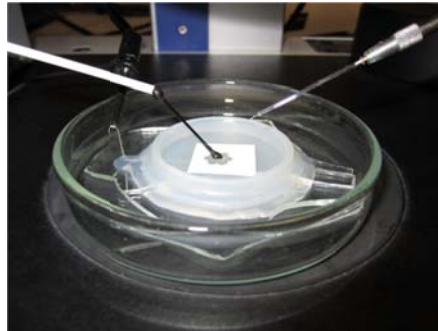


Figure 2-2 – Illustrative image showing the electroporation of chick embryo with the two electrodes and glass needle

Table 3-1 – Solution composition

Solution	Composition
10x PBS (1 liter)	80 g NaCl; 2 g KCl; 14.4 g Na ₂ PO ₄ ; 2.4 g KH ₂ PO ₄ (adjust to pH 7.4 with NaOH and bring to 1 liter with water)
Tyrode's saline (10x)	80 g NaCl; 2 g KCl; 2.71 g CaCl ₂ .2H ₂ O; 0.5 g NaH ₂ PO ₄ .2H ₂ O; 2 g MgCl ₂ .6H ₂ O; 10 g glucose to 1 l. Before use, add 20 ml of this 10x stock to 180 ml H ₂ O
Fixing Solution	4% Formaldehyde in PBS; 2mM EGTA; (adjust to pH 7.5 with NaOH)
PBT or PTW	1x PBS plus 0.1% Tween
Hybridization Solution	50% formamide; 1.3x SSC, pH 4.5 (use citric acid to pH); 5mM EDTA; 50 µg/ml yeast tRNA; 0.2% Tween 20; 0.5% CHAPS; 100 µg/ml heparin
MABT	0.5M C ₄ H ₄ O ₄ ; 744mM NaCl; 0.01% Tween 20; (adjust to pH 7.5)
NTMT	100 mM NaCl; 100 mM Tris-HCl, pH 9.5; 50 mM MgCl ₂ ; 0.1/1% Tween-20
Developing Solution	1.5ml NTMT; 4.5 µl/ml NBT (75mg/ml from Roche); 3.5 µl/ml BCIP (50mg/ml from Roche)

2.4.1. Embryo dissection and storage

Embryos presenting fluorescence were dissected (with suitable, sterilized surgery material) from the filter paper in a black Petri disk with phosphate buffer saline (PBS 1X). Then, they were placed in a 1.5ml eppendorf and immediately frozen in liquid nitrogen. Finally, the embryos were stored at -80 °C.

2.5. Whole-mount *In situ* hybridization

The principle behind **whole-mount *in situ* hybridization** (WISH) is the specific annealing of a labeled nucleic acid probe to complementary sequences (mRNA) in fixed tissue, followed by detection of the location of the probe. This method provides on one hand a spatial and temporal resolution of gene expression and on the other hand allows the detection of genetic interactions through simultaneous hybridization of up to three different probes in the same embryo.

2.5.1. Synthesis and purification of antisense RNA probes

In order to make an antisense RNA probe complementary to *hairy1* mRNA, a plasmid containing *hairy1* sequence was linearized with the restriction enzyme *HindIII* (Fermentas) and the probe was generated by *in vitro* transcription using the T7 RNA polymerase (Promega), in the presence of a digoxigenin-substituted nucleotide mix, DIG (Roche) (Palmeirim et al., 1997). The transcription product was checked on a 0,8% agarose gel. The RNA probe was precipitated by adding 200µL of Tris-EDTA buffer, 20µL LiCl 4M and 600µL Ethanol 100% and left at -80°C for 1h. The mixture was centrifuged (at 4°C) (VWR, model ct15re Himac) for 30 min at 12000rpm. The supernatant was carefully discarded and the pellet washed with 70% Ethanol, followed by centrifuging (at 4°C) for 15 min at 12000rpm. The supernatant was carefully discarded and, after being dried, the pellet was resuspended in 40 µL of Tris-EDTA buffer. The final probe was checked on a 0,8% agarose gel.

2.5.2. Embryo fixation

Embryos were fixed overnight at 4°C in fixing solution. Following removal of the fixing solution from the embryos, two 15 min washes were carried out with PTW solution. The embryos were then dehydrated for 10 min in 50% methanol/PBT and twice in 100% methanol and subsequently stored at -20°C until required.

2.5.3. Embryo pre-treatments and Hybridization

Embryos were rehydrated by washing through 75% - 50% - 25% series of Methanol/PTW solutions (for periods of 10 to 15 min) and two times in PTW. After this, embryos were treated with 10µg/ml proteinase K (Promega) in PTW. The time of incubation in min equaled the HH stage number. After that, proteinase K was removed and the embryos were briefly rinsed two times with PTW and post-fixed in 0.1% glutaraldehyde, 4% paraformaldehyde in PTW for 20 min. Embryos were then rinsed once and washed twice with PTW. The embryos were rinsed once with 1:1 PTW/hybridization mix and with 1ml hybridization mix.

Hybridization comprised two steps. First the embryos were pre-hybridized in 1ml hybridization mix for at least 1 hour at 70°C. During this period, the solution can penetrate into the cells and the optimal temperature of the hybridization is achieved. Then the hybridization mix was removed and pre-warmed hybridization mix with the previously prepared antisense RNA probe was added. Hybridization was performed overnight at 70°C.

2.5.4. Antibody incubation

After the hybridization steps, the solution was removed and the embryos were rinsed twice with prewarmed hybridization mix. The embryos were washed twice for 30 min at 70°C with 1.5ml prewarmed hybridization mix and for 10 min at 70°C with 1.5ml prewarmed 1:1 hybridization mix/MABT. Afterwards, the embryos were rinsed twice and washed once for 15 min with 1.5ml MABT solution. In order to block unspecific antibody binding sites, the embryos were incubated 1h with 1.5ml MABT containing 2% Blocking Reagent (Roche),

followed by 1.5ml MABT with 20% Goat Serum (Gibco) and 2% Blocking Reagent for a minimum of 1h at room temperature in a rocker. The previous solution was removed and replaced by fresh solution containing a dilution 1/2000 anti-Digoxigenin-AP (Roche), and incubated overnight at room temperature with smooth agitation.

2.5.5. Post-antibody washes and histochemistry

The antibody solution was removed and the embryos were rinsed in MABT three times for 5 min and washed three times for 1 h at room temperature by rolling. The embryos were then washed with NTMT solution twice for 10 min. The embryos were incubated in the developing solution in the dark, at 37°C. Once achieved the desired staining, the embryos were washed several times with PTW in order to stop the reaction and then they were photographed.

2.6. Image acquisition

Embryos were photographed using a QImaging Micro Publisher 50 RTV camera coupled to a Leica MZ16FA stereomicroscope. Images were processed using Adobe Photoshop software.

3. RESULTS

3.1. Plasmid construction for embryo electroporation

3.1.1. pCAT

In order to over-express our genes of interest in chick embryonic tissues by electroporation, an expression vector derived from pCAGGS-AFP was constructed, containing a Multiple Cloning Site (MCS), followed by an internal ribosome entry site (IRES) and the AFP gene, a modified version of the Green Fluorescent Protein – the pCAT plasmid.

The fragment encompassing an IRES-MCS region (Fig. 3-1B) was introduced in the pCAGGS-AFP plasmid and the construction was confirmed and orientation tested in eight randomly chosen colonies using two restriction enzymes: *EcoRI* and *BamHI*.

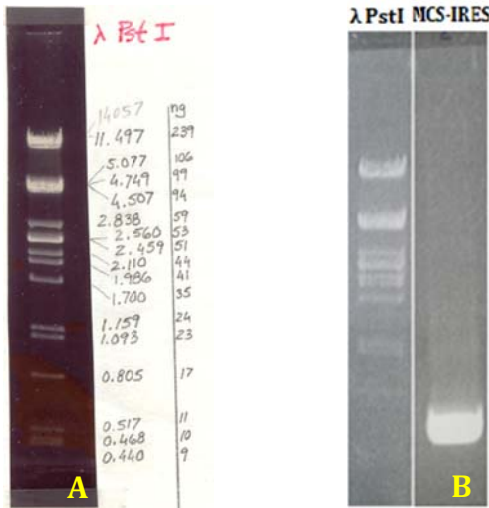


Figure 3-1 - λPstI - λDNA digested with PstI, used as a DNA molecular weight marker (A). Agarose gel with the amplified MCS-IRES fragment (B).

The *EcoRI* enzyme should linearize the plasmid producing a band of 6209 bp and this was observed in the eight colonies (Fig. 3-2B). Additional bands of higher molecular weight could also be observed, which correspond to the non-digested plasmid.

RESULTS

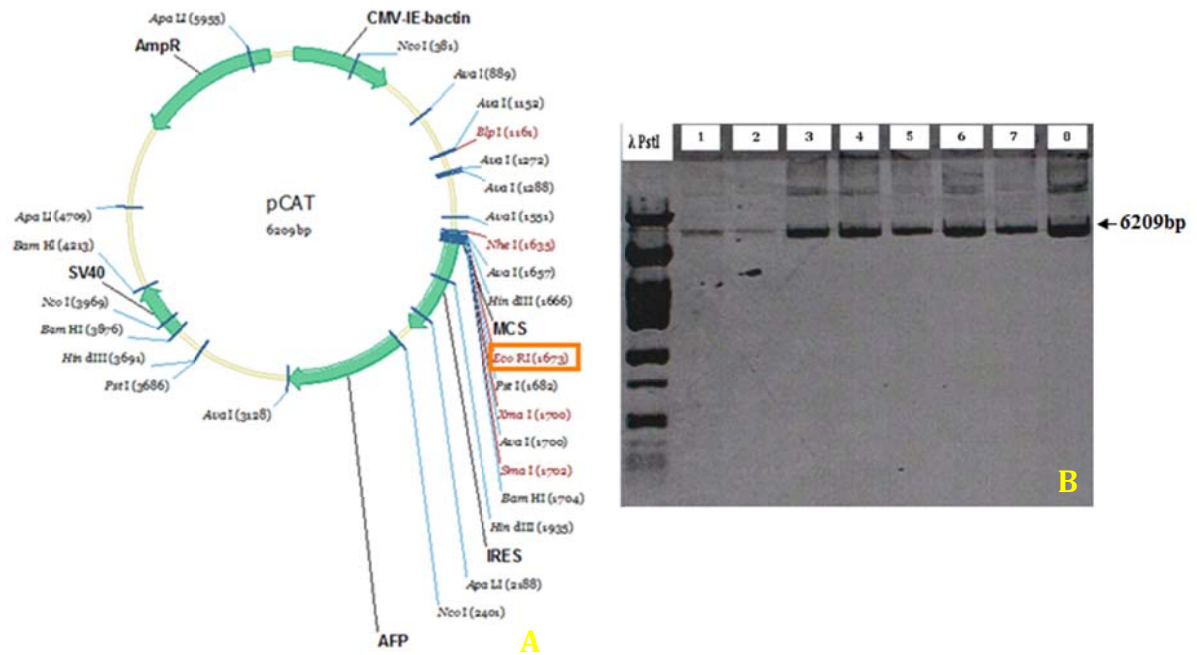


Figure 3-2 – pCAT scheme with restriction sites constructed by Vector NTI9 (Invitrogen) (A). Agarose gel electrophoresis of the 8 samples digested with *EcoRI* (B).

Upon digestion with *BamHI*, two different patterns could arise, depending on the orientation of the fragment insertion. For correct pCAT, the enzymatic digestion should produce three bands: 3700bp, 2172bp and 337bp, which occurred in colonies 2, 3, 4, 5, 7 and 8 (Fig. 3-3B). For the reverse orientation, three bands with different sizes appear: 4216bp, 1656bp and 337bp, occurring in samples 1 and 6. The band with 337bp doesn't see very well in this photo, because of the photo quality and little intensity of the band.

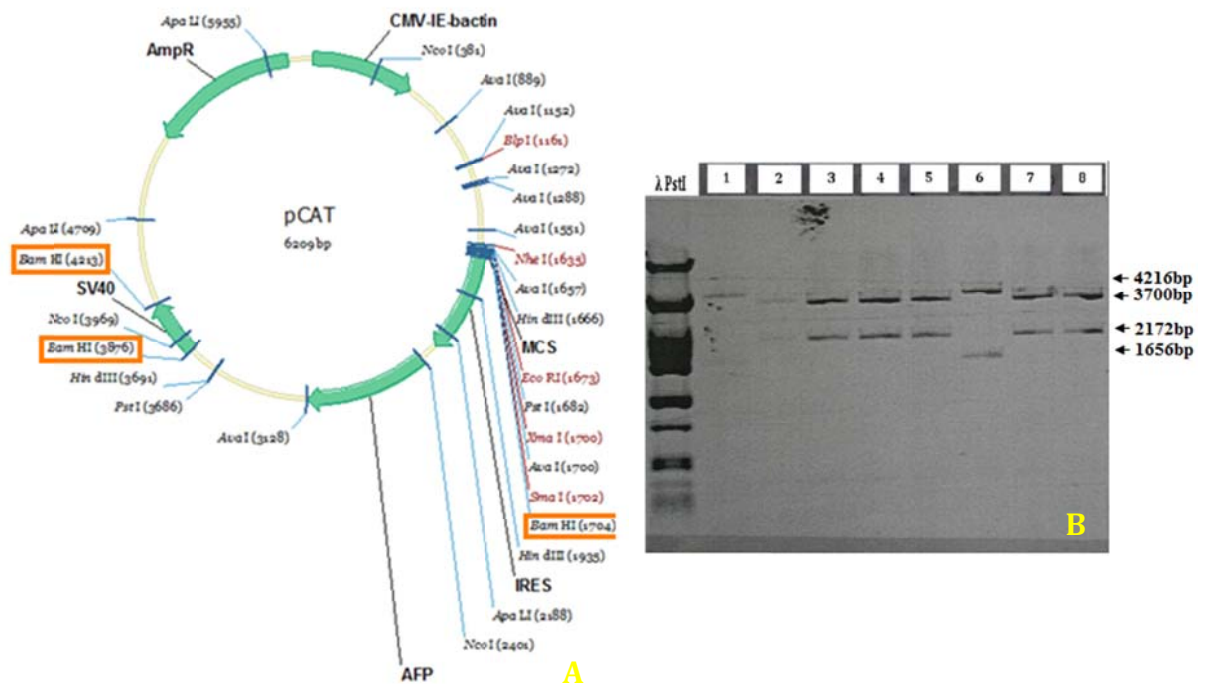


Figure 3-3 - pCAT scheme evidencing *Bam*HI restriction sites (A). Agarose gel of the 8 samples digested with *Bam*HI (B).

3.1.2. pCAT + c-Term

For pCAT+c-Term construction, the C-Terminal portion of the *hairy1* gene (Fig. 3-4A) was cloned in pCAT using two different restriction enzymes for sticky-end ligation: *Eco*RI e *Nhe*I. So, it wasn't necessary to proceed restriction enzyme digestions to check the orientation, since there's only one possibility of the fragment to be inserted into the vector, given that they have different edges. The ligated vector was then sequenced for confirmation.

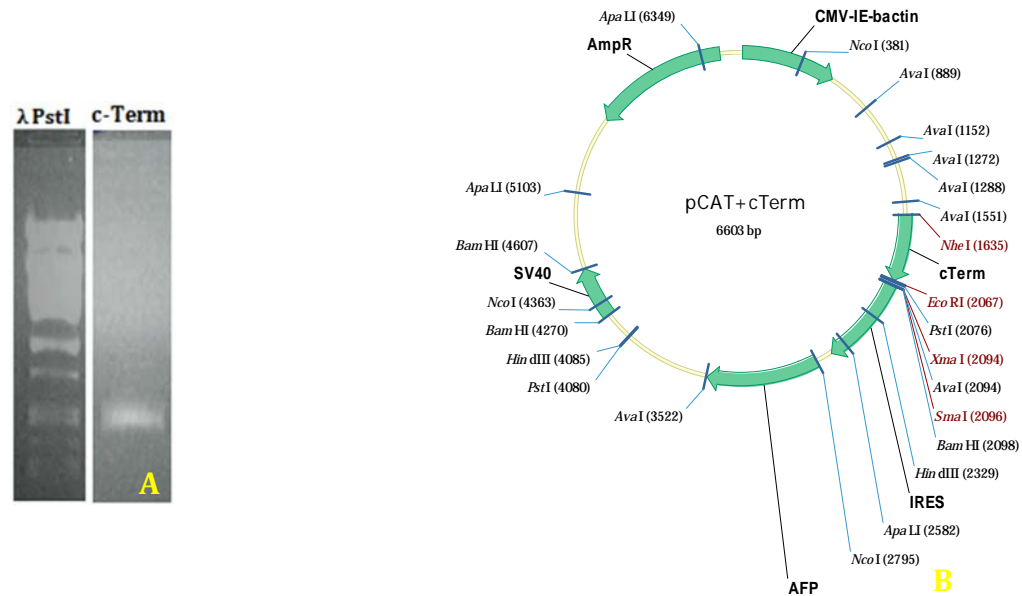


Figure 3-4 - Agarose gel with the amplified c-Term fragment comparing with the ladder λ PstI (A). pCAT+c-Term scheme with the restriction enzymes constructed by Vector NTI9 (Invitrogen)

3.1.3. pCAT + Hairy1

The pCRII-TOPO+Hairy1 plasmid was digested with *EcoRI* to obtain a fragment containing the *hairy1* gene (Fig. 3-5B). That fragment was inserted into pCAT at the *EcoRI* site. 12 randomly selected colonies were tested with *EcoRI* digestion and 7 had *hairy1* inserted (Fig. 3-5C - colonies number 1, 2, 4, 5, 9, 10 and 11).

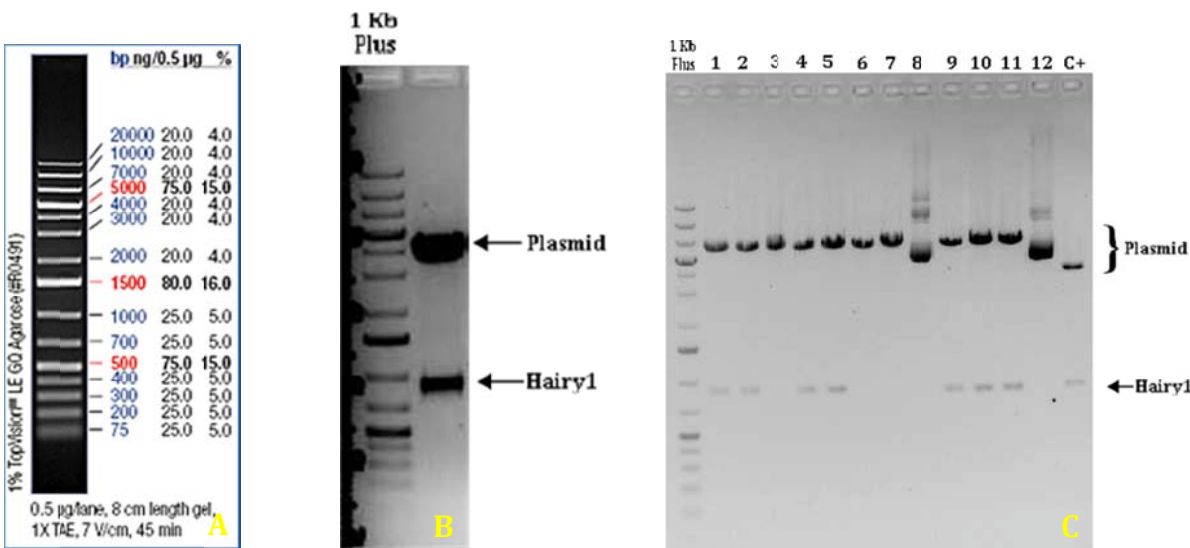


Figure 3-5 - GeneRuler's 1Kb Plus DNA Ladder specific bands (A). Agarose gel with pCRII-TOPO+Hairy1 digested with *EcoRI* (B). Agarose gel of the 12 samples digested with *EcoRI* and the positive control (C+) (C)

RESULTS

Due to the equal sticky ends of the fragment, it was necessary to distinguish which ones had the fragment inserted in the correct position (Fig. 3-6A). Five samples were obtained that amplified with the primers, indicating the correct position (Fig. 3-6B - samples 1, 4, 5, 10 and 11). Other two had the fragment inserted in a reversed position (samples 2 and 9). Samples 10 and 11 were sequenced for confirmation.

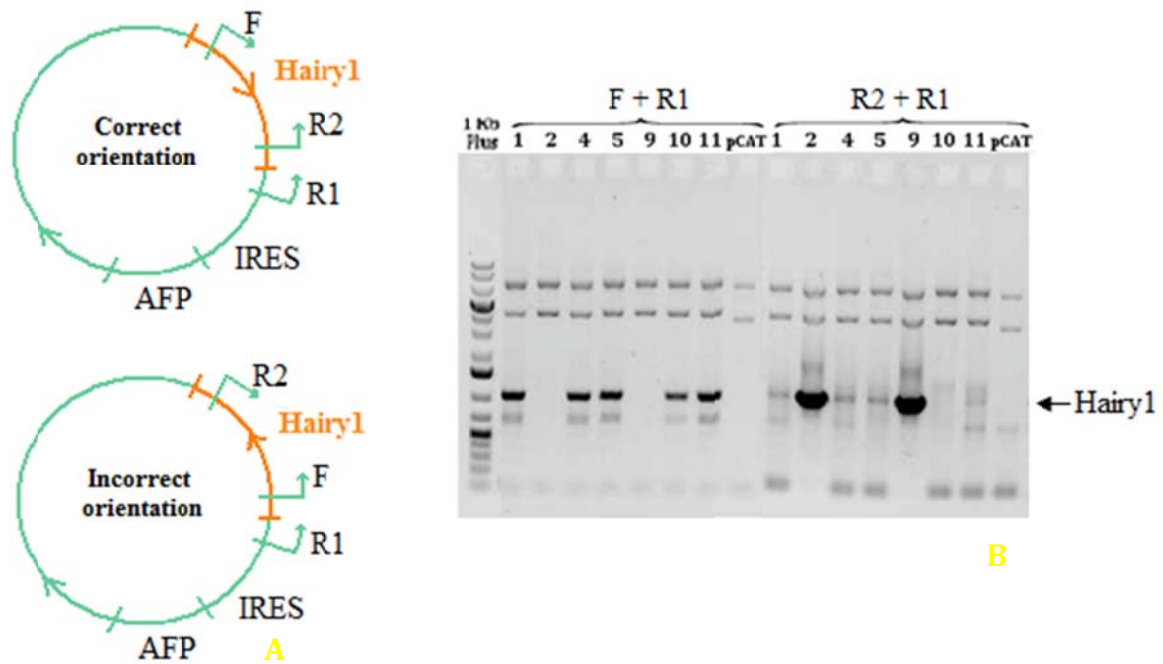


Figure 3-6 - Schematic representation of the two types of amplification according to the fragment orientation: Hairyl in correct orientation amplifies with F+R1 primers, Hairyl in incorrect orientation amplifies with R2+R1 primers (A). Agarose gel with the 7 positive samples for Hairyl insertion and negative control (pCAT) with the two different pair primers comparing with the 1Kb Plus DNA ladder (B).

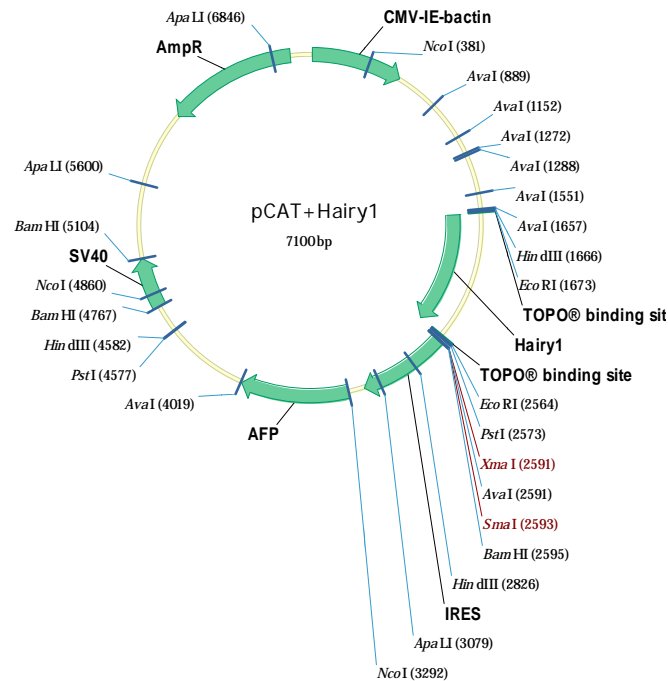


Figure 3-7 - pCAT+Hairy1 scheme with restriction sites given by Vector NTI9 (Invitrogen).

3.1.4. pCAT + Brachyury

To insert the *brachyury* gene in the pCAT, the *brachyury* ORF (Fig. 3-8A) was previously inserted into the pCRII-TOPO plasmid so that upon plasmid digestion with *Eco*RI, a fragment containing the *brachyury* gene could be obtained in 4 samples (Fig. 3-8B). The orientation of the fragment was verified by sequencing. For posterior work we chose sample 2 presenting Brachyury in a *sense* orientation where sample 4 is in *antisense* direction.

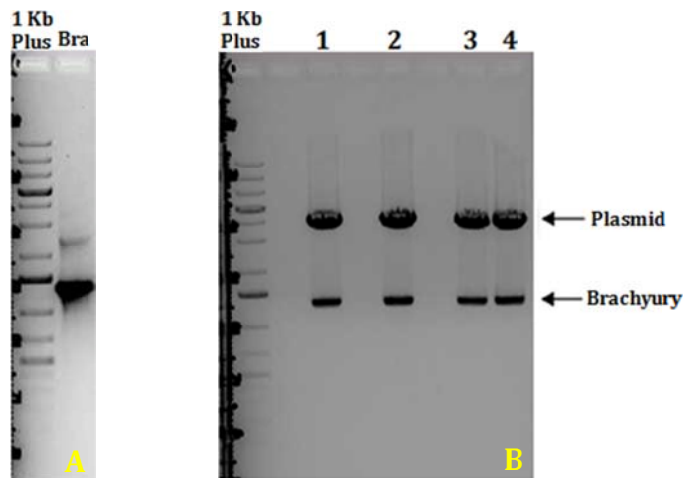


Figure 3-8 - Agarose gel electrophoresis of *Brachyury* amplified fragment (A). Electrophoresis of the 4 samples digested with *Eco*RI after insertion in TOPO plasmid (B).

RESULTS

The *brachyury* gene was obtained by the digestion of pCRII-TOPO+Brachyury with the *EcoRI* enzyme and inserted in the pCAT vector. As the edges are identical on both sides the *brachyury* can be inserted both ways. For that reason it was necessary to check their orientation. For this, two PCR reactions with different primers pairs were held (Fig. 3-9A), obtaining six samples with the correct orientation (Fig.3-9B - samples 1, 2, 3, 4, 5 and 7) and two with the incorrect orientation (samples 6 and 8). Samples 1 and 2 were sequenced for confirmation.

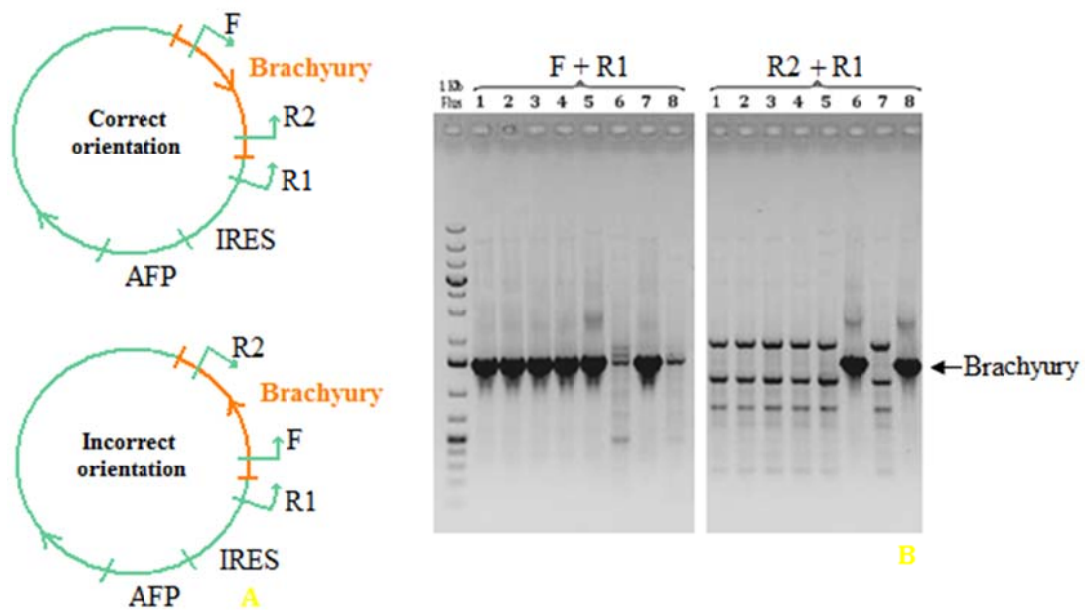


Figure 3-9 - Schematic representation of the two types of amplification according to the fragment orientation: *Brachyury* in correct orientation amplifies with F+R1 primers, *Brachyury* in incorrect orientation amplifies with R2+R1 primers (A). Agarose gel with the 8 samples amplified using the two different pairs (B).

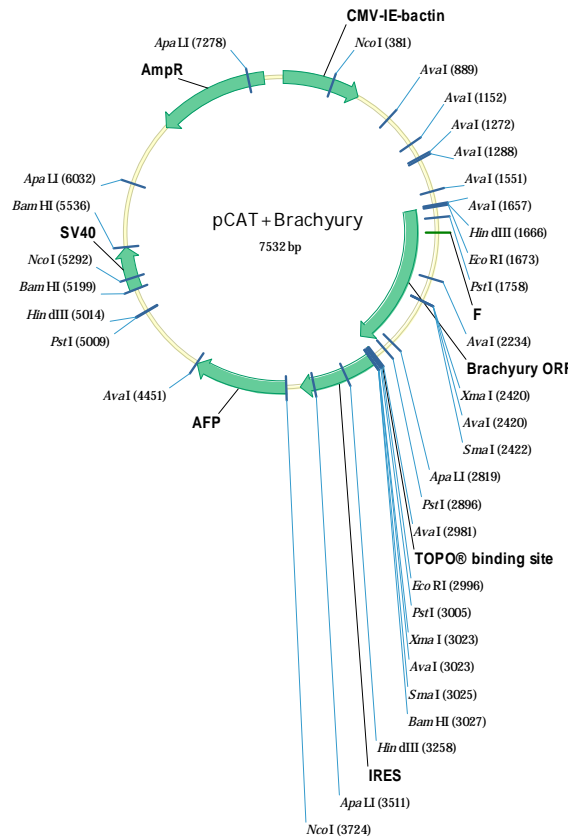


Figure 3-10 - pCAT+Brachyury scheme with restriction sites constructed using Vector NTI9 (Invitrogen).

3.2. Electroporation

3.2.1. Optimization of chick electroporation conditions

The electroporation technique was a new method introduced in the lab, and it was necessary to proceed to its optimization. For this, 441 3-4HH embryos were electroporated with empty vectors in order to test several electroporation conditions. This worked allowed the conclusions described below.

- Voltage applied by the electroporator: values higher than 10 volts are lethal to the embryos, while values below 10 volts are many times insufficient and no fluorescence can be observed.

- The use of ultra-pure distilled water and sterilized material is of dire need, for its non-fulfillment may introduce contaminations compromising all the methodology.
- The type of fluorescence chosen to the plasmid was based simply on the best visualization of the color green (AFP) (Fig. 3-11B) opposed to color red (RFP) (Fig. 3-11C), since there was no toxicity differences in the embryo using either one.

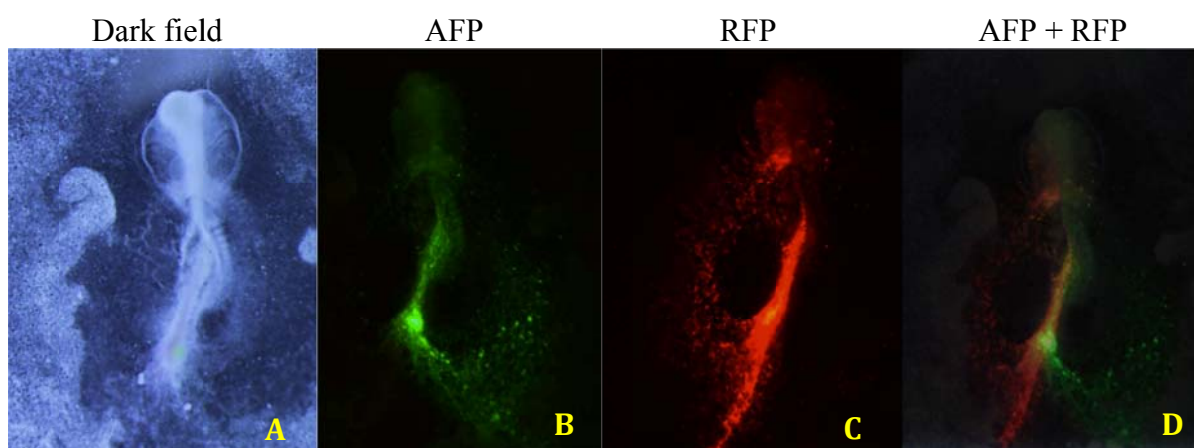


Figure 3-11 - Images of an embryo with different types of fluorescence: AFP (B), RFP (C) and both AFP+RFP (D)

- The plasmid concentration was also tested. Fluorescence was obtained without apparent toxicity using all the concentrations tested: 1.55 $\mu\text{g}/\mu\text{l}$, 1.02 $\mu\text{g}/\mu\text{l}$, 0.56 $\mu\text{g}/\mu\text{l}$, 0.3 $\mu\text{g}/\mu\text{l}$, 0.21 $\mu\text{g}/\mu\text{l}$ and 0.13 $\mu\text{g}/\mu\text{l}$. In general, an approximated value of 0.3 $\mu\text{g}/\mu\text{l}$ were chosen to inject, for being a value that presented fluorescence, it didn't present any real toxicity and it was the value directly obtained from the DNA extraction by the MiniPrep kit.

3.2.2. Confirmation of plasmid-driven gene over-expression

To confirm the plasmids were over-expressing the gene of interest, electroporated embryos presenting fluorescence were processed for *in situ* hybridization with either Hairy1 or Brachyury probe (Fig. 3-12 and Fig. 3-13).

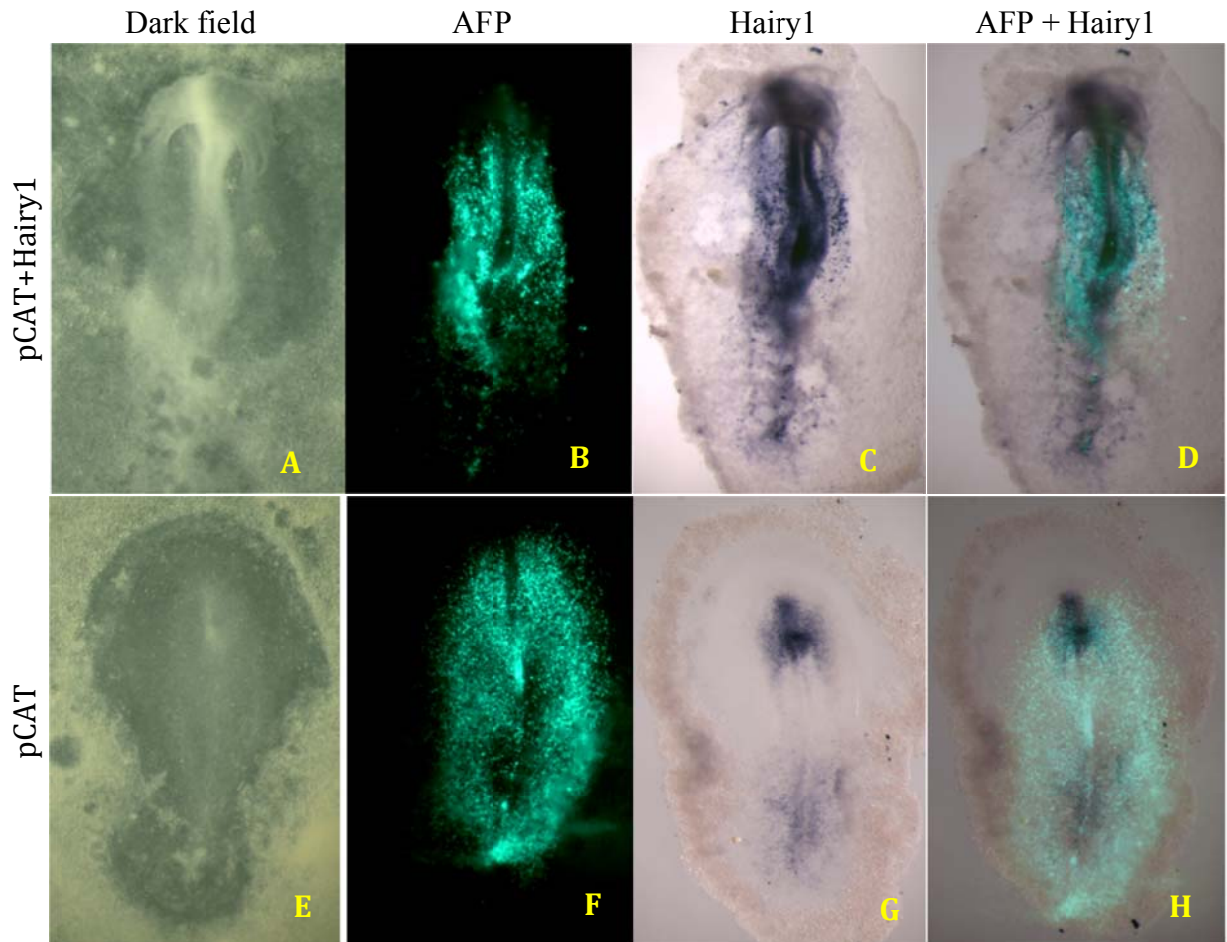


Figure 3-12 – Embryo electroporated with pCAT+Hairy1 observed in: dark field (A), AFP fluorescence (B), Hairy1 staining by *in situ* hybridization (C) and an overlap image of AFP and Hairy1 (D). Embryo electroporated with pCAT observed in: dark field (E), AFP fluorescence (F), Hairy1 staining by *in situ* hybridization (G) and an overlap image of AFP and Hairy1 (H).

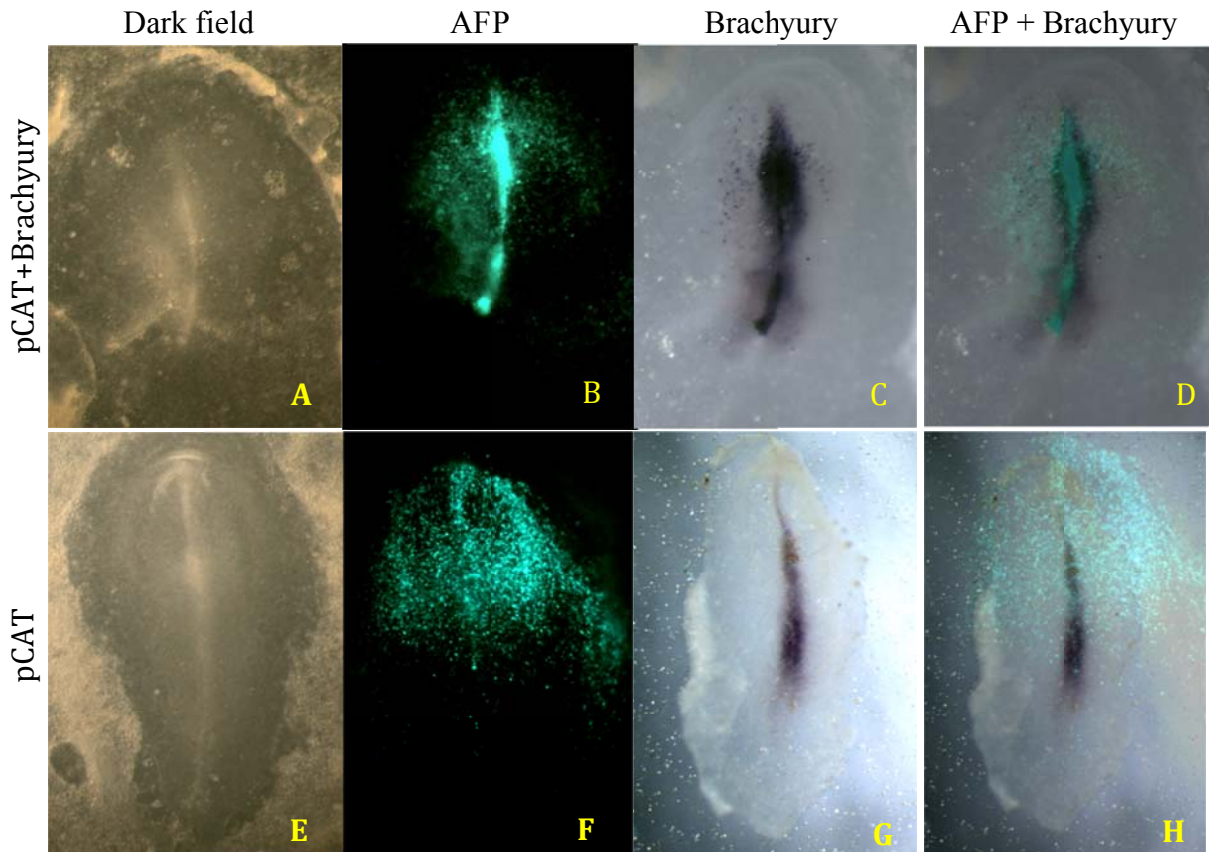


Figure 3-13 - Embryo electroporated with pCAT+Brachyury observed in: dark field (A), AFP fluorescence (B), Brachyury staining by *in situ* hybridization (C) and an overlap image of AFP and Brachyury (D). Embryo electroporated with pCAT observed in: dark field (E), AFP fluorescence (F), Brachyury staining by *in situ* hybridization (G) and an overlap image of AFP and Brachyury (H).

The analysis of *in situ* hybridization staining on the embryos previously electroporated with pCAT+Hairy1 and pCAT+Brachyury revealed a pattern corresponding to the endogenous *hairy1* or *brachyury* expression, respectively, plus a “salt-and-pepper”-like staining that matched and overlapped with the AFP fluorescence. This indicates that an over-expression of both AFP and Hairy1 or Brachyury has been achieved.

3.2.3. Effect of the local of injection in the over-expression pattern

The plasmids with the gene of interest were introduced in the embryo by microinjection between the hypoblast and the vitelline membrane in two different ways. The first one, through a more crafty technique, in which the needle was introduced in a silicone tube, the pressure was performed with the mouth and two or three micro-injections were performed at the posterior part of the embryo, behind the primitive streak (Fig. 3-14A, B, C,

D). Alternatively, a micro-injector with automatic manipulation was used and six to eight injections were performed at the anterior part of the embryo, on both sides of the primitive streak (Fig. 3-14E, F, G, H). This difference in the localization of the injection also resulted in different localization of the fluorescence.

According to the DNA injection site different expression patterns of plasmid-driven fluorescence were obtained. When injected in the posterior part of the primitive streak the fluorescence also appeared in that location, poorly contributed to the PSM (Fig. 3-14A, B, C, D). When it was injected in the anterior part of the embryo, right below the Hensen's Node and lateral to the anterior tip of primitive streak, the fluorescence appeared dispersed in neural plate of the embryo and in the notochord (Fig. 3-14E, F, G, H). If the embryo was injected down to 60% of the primitive streak, all the PSM and somites would be fluorescent (Fig. 3-14I, J, K, L). Last, if the injection was performed down to 20% of the primitive streak, the entire embryo is labeled with the exception of a region containing the PS and an area surrounding it (Fig. 3-14M, N, O, P). As our objective was to electroporate the cells of the paraxial mesoderm, the site of injection presented in Fig. 3-14I, J, K, L was further chosen for being the most effective.

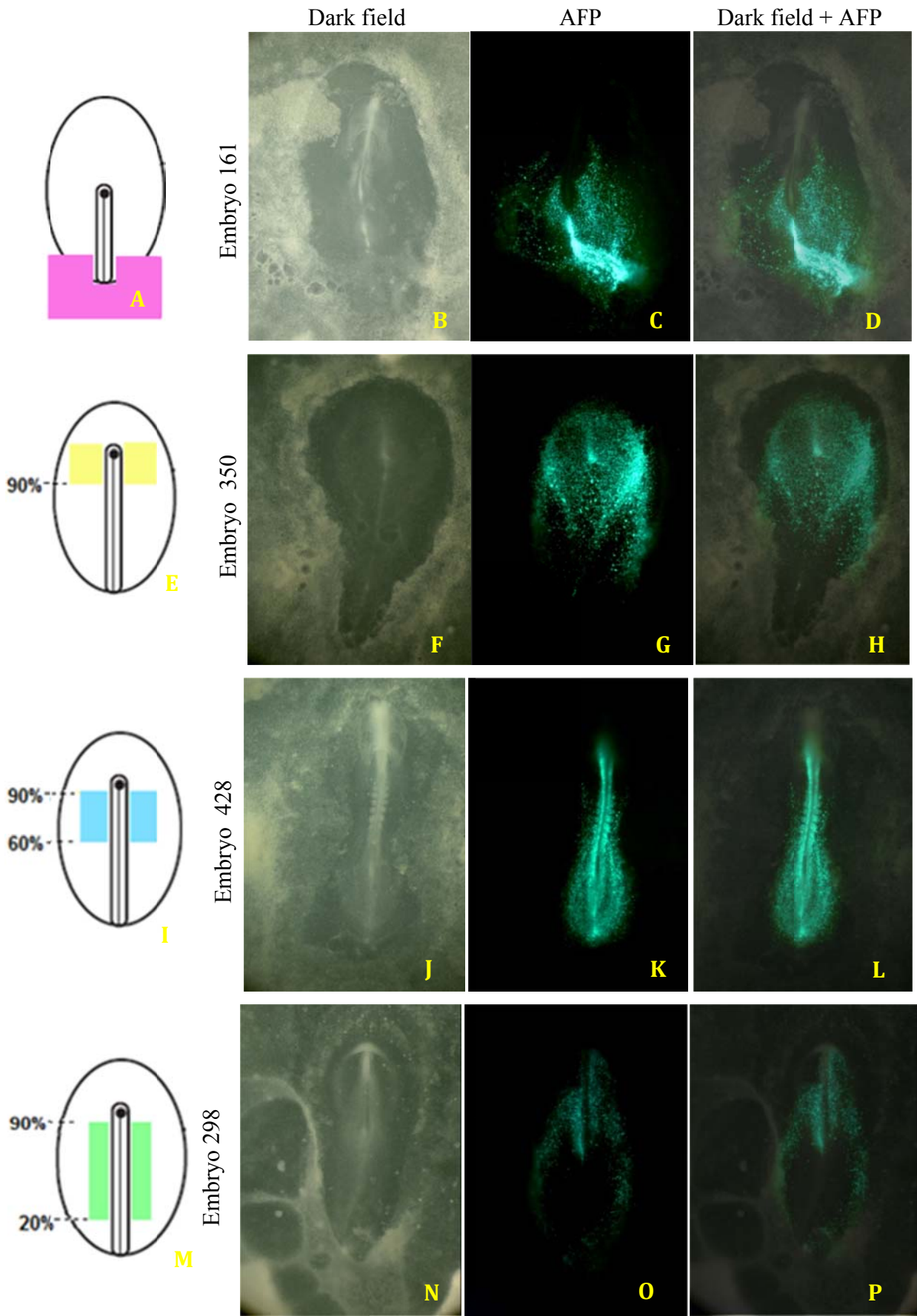
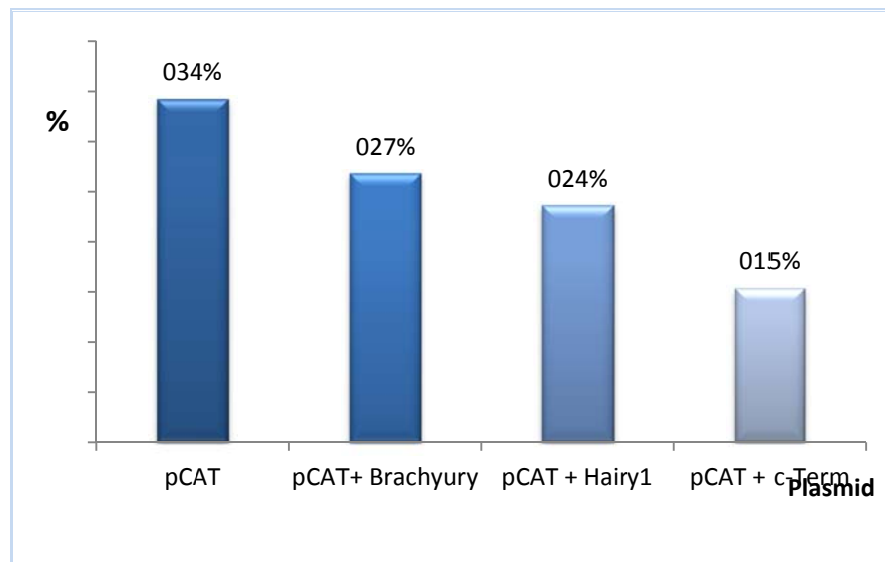


Figure 3-14 - Schematic representation of the local of injection (A, E, I, M). Embryos in three different staining: dark field (B, F, J, N), AFP fluorescence (C, G, K, O) and overlap of both dark field and AFP fluorescence (D, H, L, P)

3.2.4. Distribution of the electroporated embryos

From the 463 electroporated embryos, 151 (32.6%) presented fluorescence. 123 of these were collected for posterior quantitative RT-PCR and 28 for whole-mount *in situ* hybridization. The distribution of the 123 electroporated embryos with the different plasmids is schematized in Graphic 3-1.



Graphic 3-1- Distribution of the 123 embryos (percentage of embryos) electroporated with the different plasmids: pCAT, pCAT+Brachyury, pCAT+ Hairy1 and pCAT+c-Term.

3.2.5. Hairy1 over-expression phenotype

Analysing the results obtained, and independently of the different locals of plasmid injection, we didn't observe significant differences in embryo death in Hairy1/Brachyury/c-Term electroporated embryos comparing with the controls. Nevertheless, 56% presented some sort of malformation.

From the embryos electroporated with the *hairy1* gene in the PSM (n=10), four did not develop beyond the stage at which they were electroporated and six (60%) had grown and developed. One of these presented normal development, but it only grew until st. 6HH, so we don't know if a delayed phenotype would be evidenced later. The other 5 embryos, corresponding to a total of 83%, presented less somites than expected when compared to the normal head development expecting 2-4 somites (Fig. 3-15). This distribution can be confirmed in the Diagram 3-1.

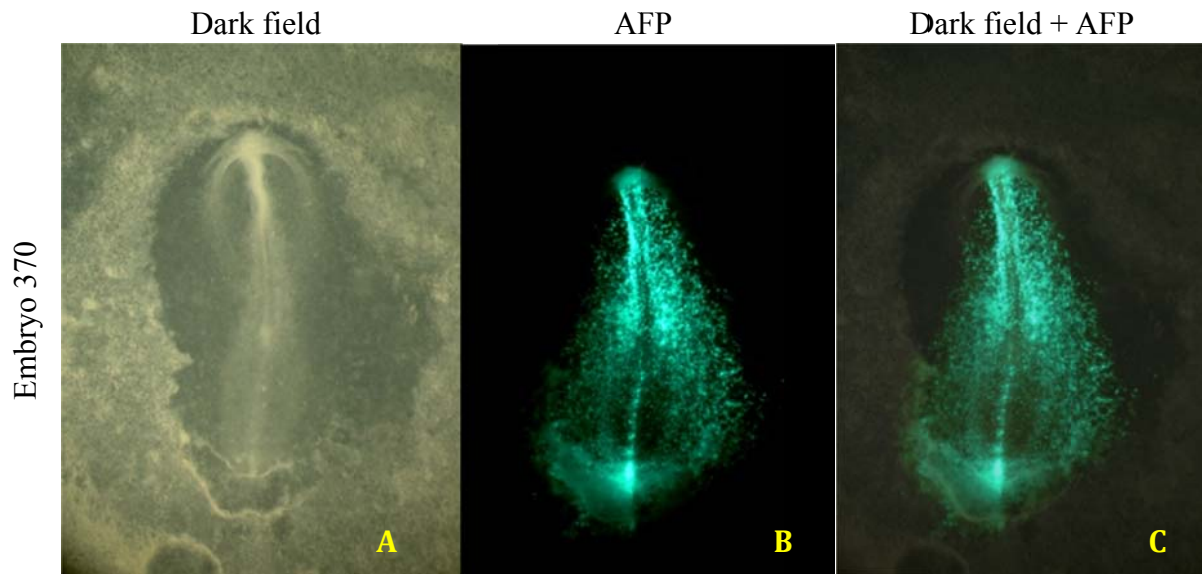


Figure 3-15 – Example of an embryo with the characteristic phenotype with less number of somites comparing with the head development. Embryo 370 in three different staining: dark field (A), AFP fluorescence (B) and overlap of both dark field and AFP fluorescence (C)

3.2.6. Brachyury over-expression phenotype

From a total of 25 embryos electroporated with *brachyury* in the PSM, 18 (72%) had grown during the incubation period. From these, four embryos (22%) were greatly delayed in development and 11 embryos, corresponding to 61%, had the same phenotype (less number of somites) that was observed in the embryos electroporated with *hairy1* (Fig. 3-16 embryo 404). This distribution can be confirmed in the Diagram 3-1.

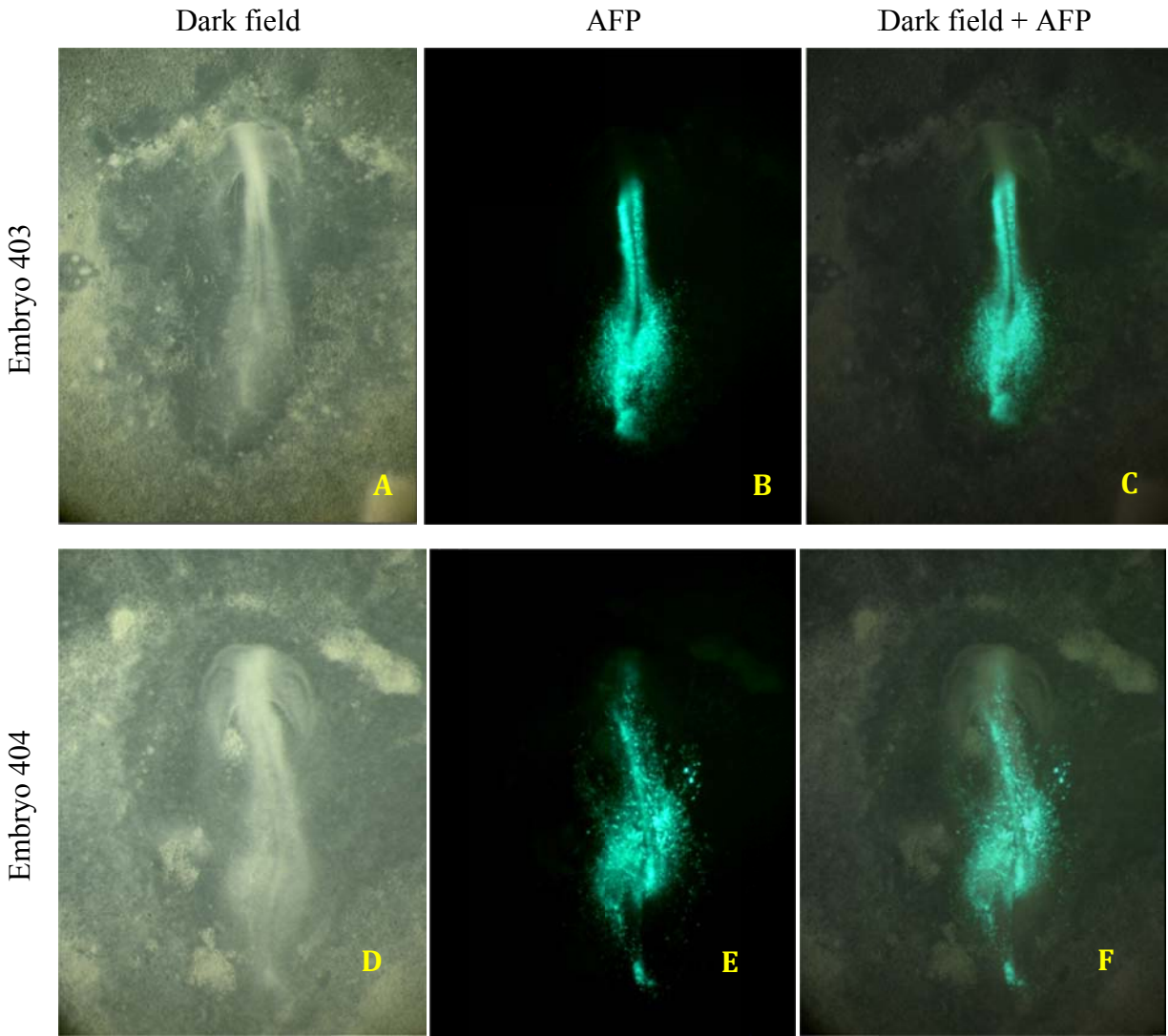
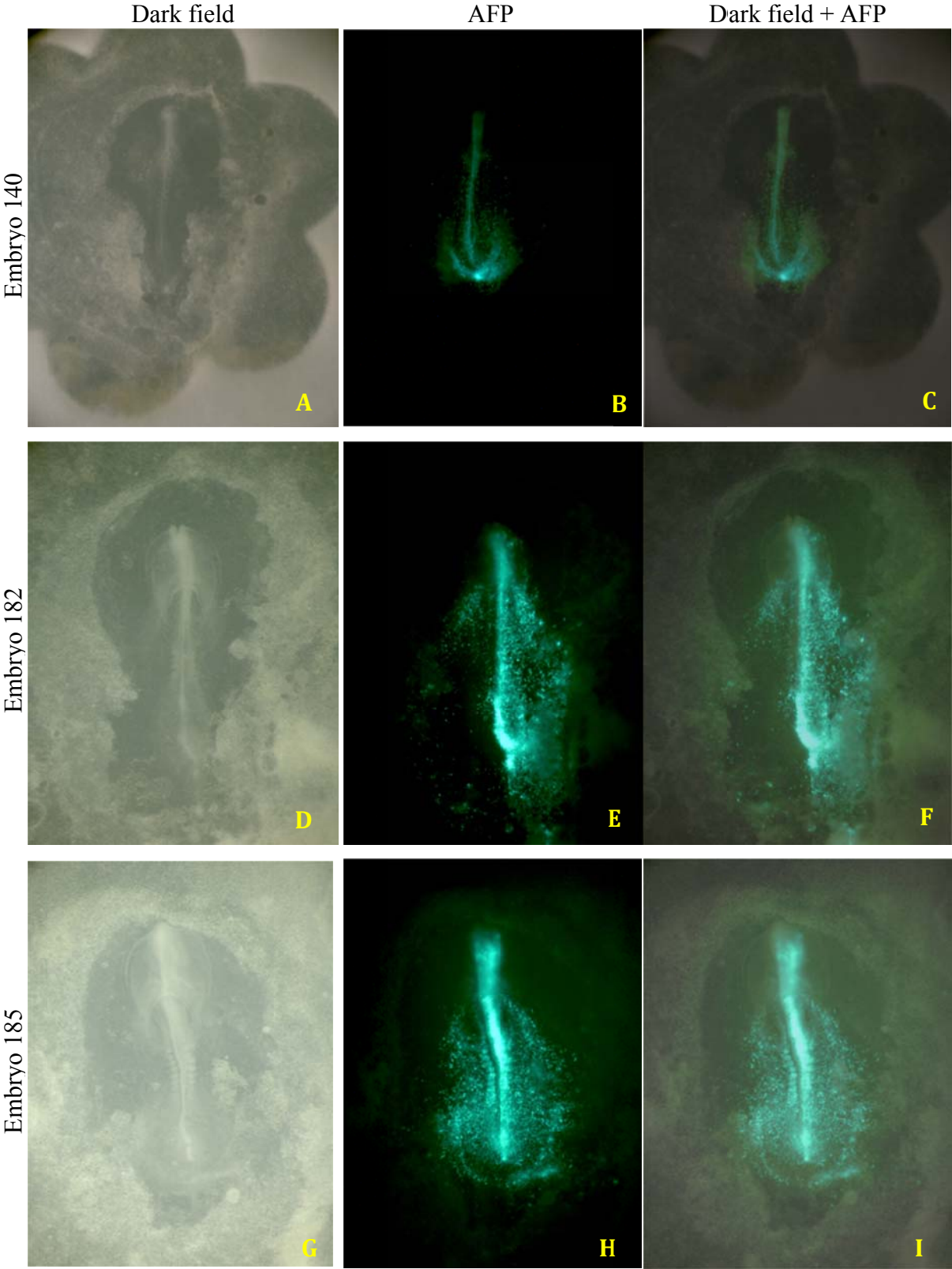


Figure 3-16 – Comparison of a normal embryo (embryo 403) with an example of an embryo with the characteristic phenotype with less number of somites comparing with the head development (embryo 404). Embryos in three different staining: dark field (A and D), AFP fluorescence (B and E) and overlap of both dark field and AFP fluorescence (C and F).

3.2.7. c-Term over-expression phenotype

From a total of 16 embryos electroporated with c-Term in the PSM, 10 embryos (63%) had grown and developed. From these, four embryos with normal development were obtained, three presented different types of malformations and three embryos, corresponding to 30%, had the some phenotype (less number of somites) that was observed when the embryos were electroporated with *hairy1* or *brachyury* (Fig. 3-17 embryos 182, 185 and 162). This distribution can be confirmed in the Diagram table 3-1.



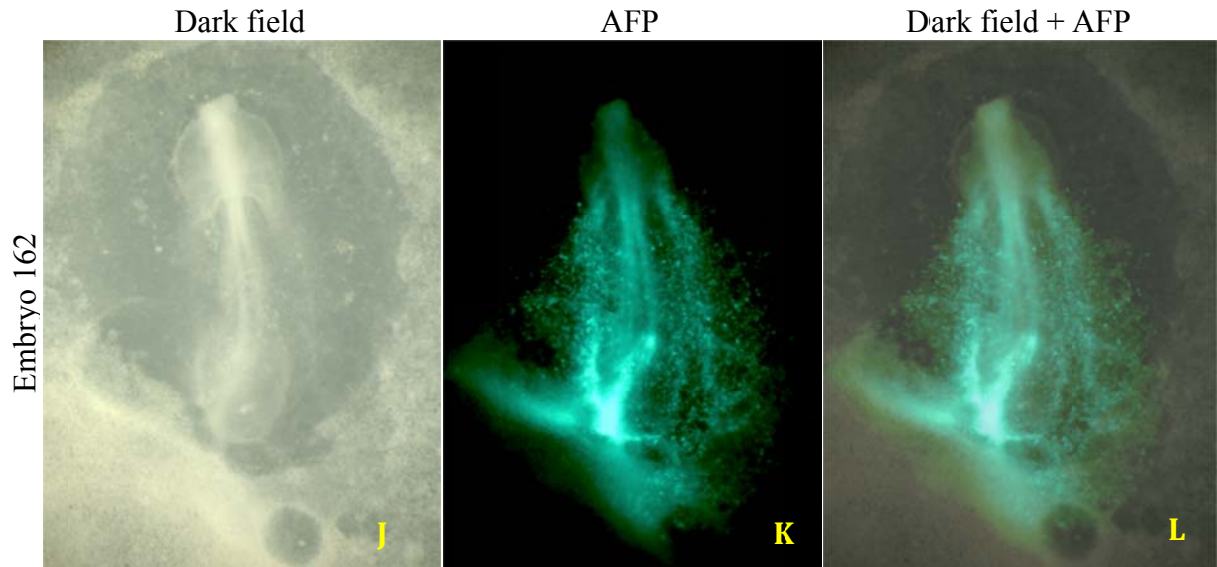


Figure 3-17 - Comparison of a normal embryo (embryo 140) with three types of severity of the characteristic phenotype with less number of somites comparing with the head development (embryos 182, 185 and 162). Embryos in three different staining: dark field (A, D, G and J), AFP fluorescence (B, E, H and K) and overlap of both dark field and AFP fluorescence (C, F, I and L).



4. DISCUSSION

4.1. Prospective-PSM is located in the anterior third of the primitive streak

Observing our results, we verify that embryos electroporated in the posterior part of the primitive streak present very few morphologic alterations. Contrastingly, embryos electroporated in the anterior domain were the ones where greater alterations were observed, perturbing the normal development of the embryo. As the site of plasmid injection and electroporation goes down along the primitive streak, a spread of extra-embryonic staining appears in the embryo.

Considering the site of plasmid injection and electroporation on 3⁺/4 HH embryos and the domains presenting fluorescence after incubation, our results are in accordance to the fate maps described in the literature (Iimura and Pourquié, 2006). The anteriormost epiblast, which lies adjacent to the node and the anterior primitive streak, essentially gave rise to neural and ectodermal derivatives. The epiblast region lateral to the primitive streak between 90% - 60% levels of streak extension contained paraxial mesoderm precursors. Posterior to this level of the streak, the epiblast derivatives were essentially found in the lateral plate or extra-embryonic mesoderm.

It was previously described that activation of the *hoxb* cluster is first initiated in the paraxial mesoderm precursors located in the epiblast in a collinear fashion before their ingression into the primitive streak (Iimura and Pourquié, 2006). For that reason, we considered that the preferential local of injection for gene over-expression in these cells is located between 90% and 60% levels of primitive streak extension lateral to the streak.

4.2. Electroporation drives to a “salt-and-pepper” pattern of Hairy1/Brachyury over-expression

We observed that electroporation produces a non-continuous pattern of mRNA expression, but rather a “salt-and-pepper” pattern. This characteristic is due to the fact that single cells are randomly electroporated, instead of the whole tissue. This is very well observed in the fluorescence and *in situ* hybridization images. Hairy1 expression appeared first of all in a continuous form in the embryo that corresponded to the endogenous gene.

After this, the exogenous Hairy1 produced only in the cells that received the plasmid, was also evidenced showing a typical “salt-and-pepper” pattern. As for Brachyury expression, both endogenous and exogenous gene expression was revealed simultaneously, maybe because the electroporation was performed with a higher plasmid concentration or there may be differences in mRNA detection by *in situ* hybridization between Hairy1 and Brachyury. Finally, embryos electroporated with the control plasmid (pCAT) only presented Hairy1 and Brachyury staining corresponding to the endogenous gene. All the GFP fluorescence observed in these embryos didn’t match the staining obtained when *in situ* hybridization was performed with either Hairy1 or Brachyury, as expected.

4.3. Both Hairy1 and Brachyury over-expression in PSM cells perturbs somite formation

Observing the embryos electroporated with Hairy1, Brachyury and c-Term, we obtained a high percentage of embryos (73%) with a common feature: a reduced number of somites than were expected when comparing with their normal head development. The reason for this phenomenon is not clear. To further clarify this, we should increase the number of electroporated embryos or let them grow more to understand if impairment or delay in the somite formation are occurring. In the embryos presenting fluorescence and a normal number of somites, the part of the embryo that was efficiently electroporated seemed to be the neural tube instead of the PSM. The difference in the tissue which was the target of electroporation could explain the normal number of somites, since it didn’t affect the cells that are responsible for somite formation.

This phenotype appears in the embryos electroporated with any one of the three plasmids (83% in Hairy1, 61% in Brachyury and 30% in c-Term), suggesting a relationship between Hairy1 and Brachyury. These two proteins have been shown to interact with each other to form a dimer (unpublished results). When an over-expression of one or other protein is performed, we perturb this ligation and alteration in somite formation occurs. Although the high level of malformations in control embryos should be clarified, only one case presented a similar phenotype with less somites, indicating that this phenotype is most probably specific and due to protein over-expression.

4.4. c-Term over-expression leads to a mild, dose-dependent somitic phenotype

c-Term electroporated embryos present a greater variety of phenotypes, in which 40% of the grown embryos are normal.

The three embryos with less number of somites (#162, 182 and 185), corresponding to the 30% of the grown embryos, are different from each other, showing a gradient of severity on somite formation according to the amount of plasmid electroporated. This gradient suggests a dose-dependent mechanism in somite formation. By ascending order of severity, in embryo #182 only the right side was electroporated and presented somite malformation when compared to the left side, that presented normal somites. Embryo #185 also had the right side more electroporated than the left and fewer, malformed somites were observed. Finally, embryo #162 is the one that presented a higher level of malformations where no somites were observed. In any case, these alterations in somite formation are less dramatic than the phenotype obtained in *Hairy1* or *Brachyury* electroporated embryos, since some somites can be observed.

4.5. Conclusions and future perspectives

In summary, the results presented in this work evidence the utility of electroporation in allowing gene over-expression in the chick embryo and sustain the hypothesis that *Hairy1* and *Brachyury* proteins exert a biological effect as a protein dimer since the same phenotype in somite formation was obtained when embryos were electroporated with either gene.

Brachyury is known to be required from *Hox* gene expression initiation. To assess if *Hairy1* or *Brachyury* over-expression affect *Hox* gene expression, quantitative RT-PCR experiments will be performed in relevant embryos obtained throughout this work. With that purpose, all the embryos were frozen and carefully manipulated with RNase free material. Different genes will be tested, namely *hairy1* and *brachyury* to quantify mRNA over-expression and the *Hox* genes from cluster B, *hoxb1*, *hoxb2*, *hoxb3*, *hoxb5*, *hoxb8*, *hoxb9* and *hoxb13*. The mRNA levels obtained will be normalized to appropriate reference genes.

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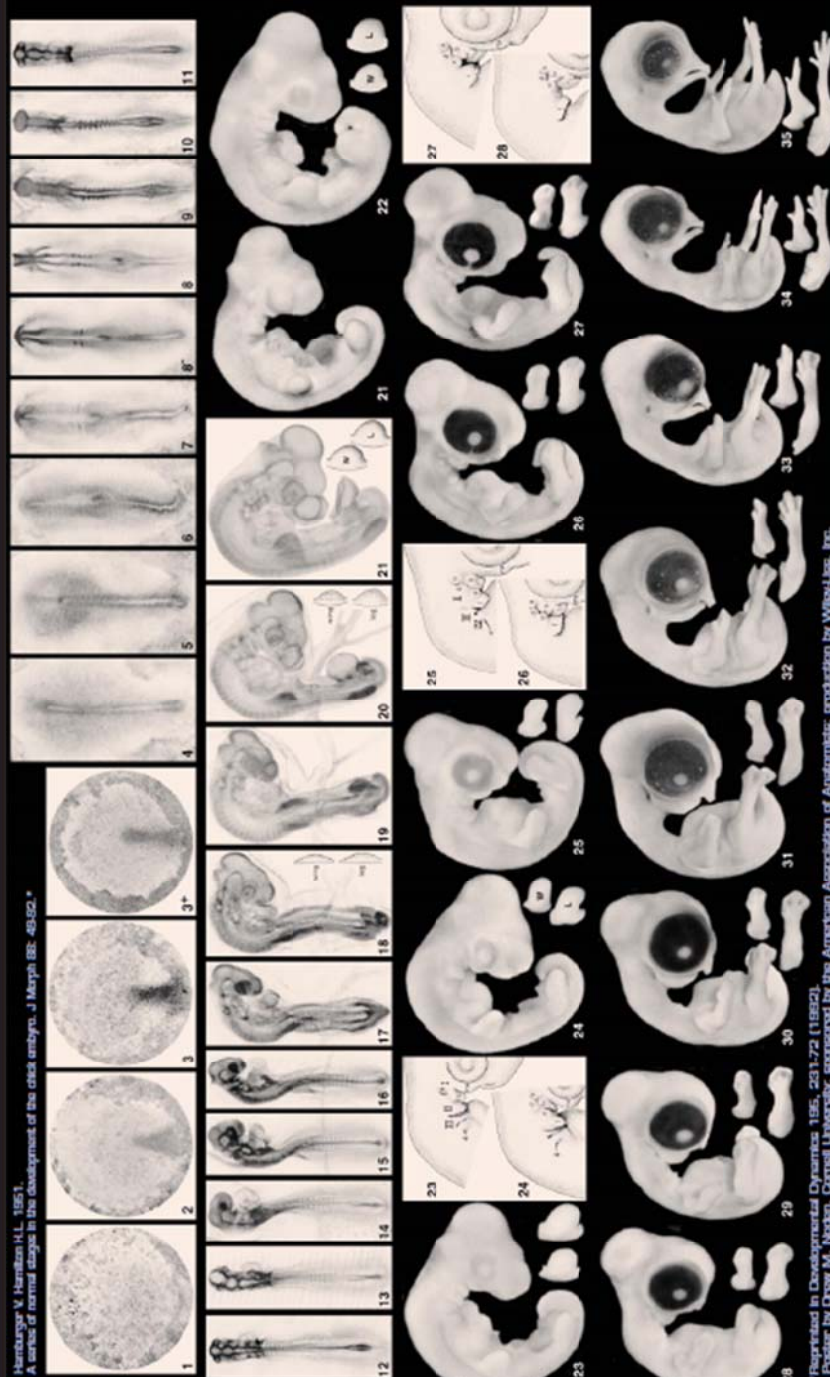
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6. APPENDIX

In Memory of Viktor Hamburger: 1900-2001

Hamburger V, Hamilton H.L. 1951.
A series of normal stages in the development of the chick embryo. J Morph 88: 49-52. *



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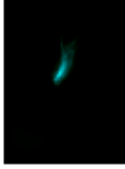



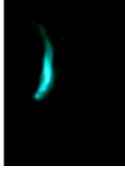

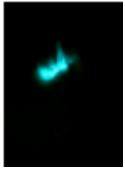

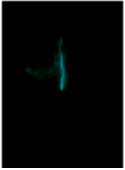

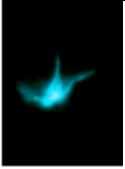

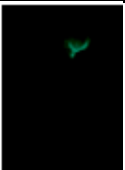

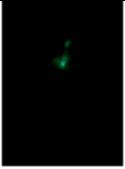
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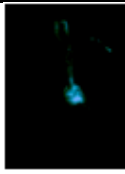

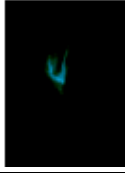

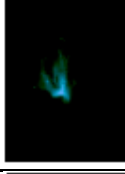



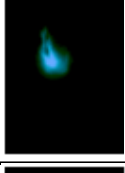

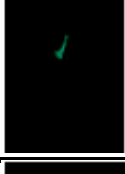

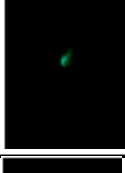

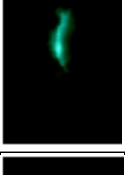

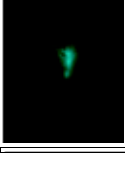
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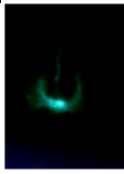

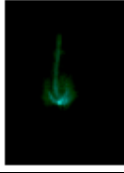



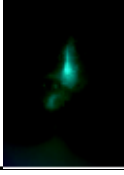

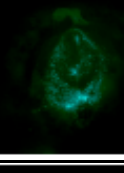

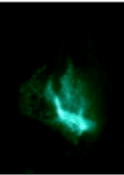

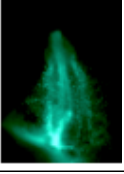

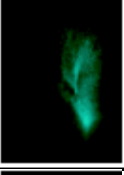

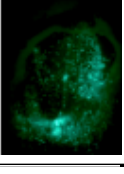
Electroporated embryos list

Date	ID	Plasmid	stage	Incub Time	Final stage	Little growth	Malformed	Local expression	Dark filed	AFP
10-02-10	31	pCAT	3	21h45	9-			PSM		
	35		3+	19h30	8?		x	P		
	36		4	17h	9-			PSM		
	38		4	17h	8?		x	P		
	47		4	20h30	8		x	PSM		
	52		5	17h	9+			P		
	56		3	22h	3+	x		PSM		
	58		3	22h	3+	x		PSM		


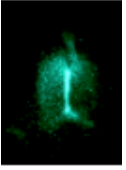

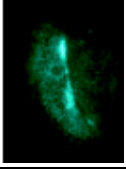

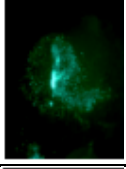

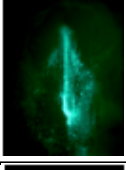

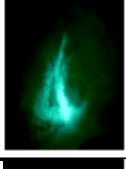

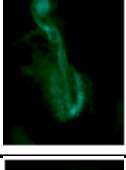

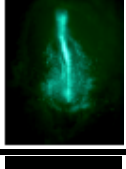

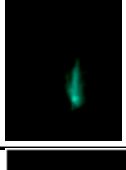

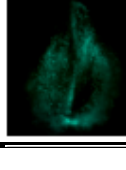
APPENDIX- 2

	63		4	21h	8?			P		
	64		4	21h	9-			P		
	65		4	21h	5	x		P		
	75		4	15h	8			P		
	76		4	15h	8		x	P		
	106		3+	18h	4	x		PSM		
19-02-10	116	pCAT+ cTerm	3+	19h	4-			PSM		
	119		4	18h20	9+		x	PSM		
	123		4	17h30	7?		x	P		


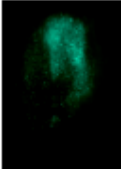

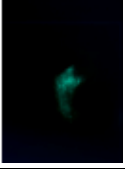

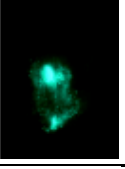

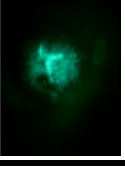

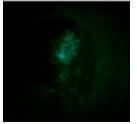

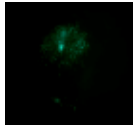

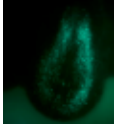



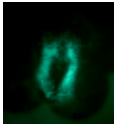
APPENDIX- 2

26-02-10	135	pCAT+ cTerm	3+	19h	8			P		
	140		4	18h	9			PSM		
	142		4	16h	8-			PSM		
	152		3+	17h	3+	x		PSM		
	158		3+	16h	7			PSM		
26-03-10	161		3+	20h	8?		x	P		
	162		3+	20h	8?		gs	PSM		
	163		3+	19h	4	x		PSM		
	164		3+	19h	5	x		PSM		


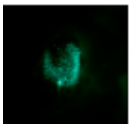

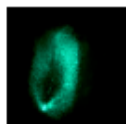

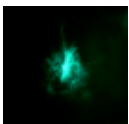

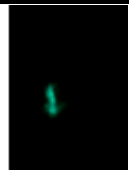

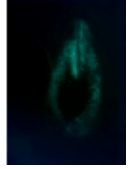

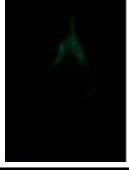






APPENDIX- 2

	165	pCAT+ cTerm	3+	18h	3+	x		PSM		
	166		4	18h	4	x		PSM		
07-04-10	181		3+	20h	7?		x	PSM		
	182		4	20h	8-		gs	PSM		
	183		4	19h	?		x	PSM		
	184		5	18h30	10			PSM		
	185		3+	18h	9		gs	PSM		
20-04-10	207		4-	13h	?		x	P		
	208		3+	16h	6			PSM		


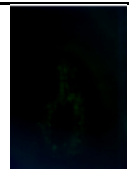

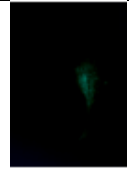







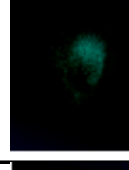

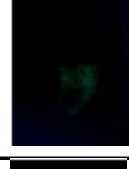

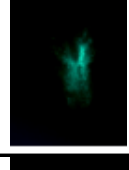


APPENDIX- 2

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	223		3+	13h	4	x		PSM		
	227		3+	15h	3+	x		PSM		
	228		3	15h	3+	x		PSM		
30-04-10	248	pCAT	3	16h40	3+	x		A		
	249		3	16h20	5		late	A		
06-05-2010	270		3+	13h30	6			E		
	273		3+	16h	5	x		PSM		
	274		3+	15h45	5		late	E		


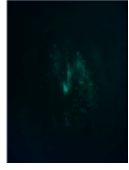

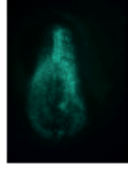

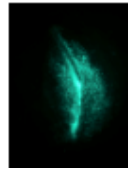

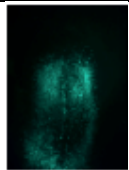

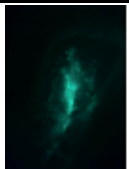

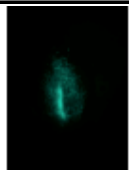

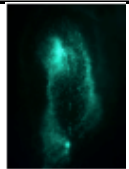

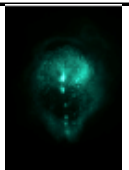

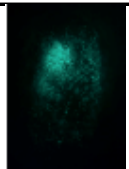
APPENDIX- 2

	275	pCAT	3	15h15	5		x	A		
	276		4	12h15	5		late	E		
	277		4	11h30	6+		x	PSM		
14-05-10	290	pCAT+ Hairy1	3+	12h40	?	x		PSM		
26-05-10	298	pCAT+ Hairy1	3+	14h20	6+			E		
	299		3+	14h20	7+			E		
	300		4-	14h	7+			E		
	301		4-	16h	5		x	A		
	304		4-	14h	5+			A		




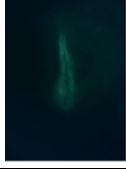







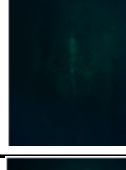


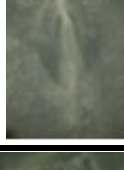
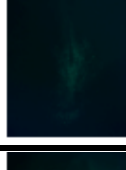

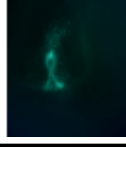
APPENDIX- 2

	305		4	14h	6			E		
	307		3	14h	4	x		A		
27-05-10	308	pCAT	3+	14h30	5	x		A		
	309		3+	17h15	5		late	A		
	310		3+	17h30	5+		late	E		
	311		3+	17h45	4	x		A		
	312		3+	17h	4	x		E		
	314		4-	12h30	» 6		x	PSM		
	315		3+	16h	6+		gs	PSM		


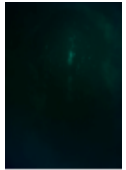



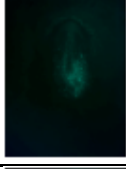

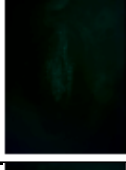

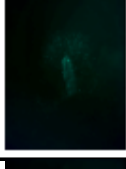

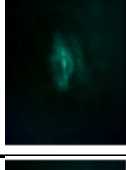

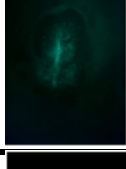

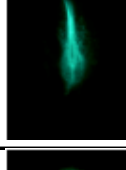

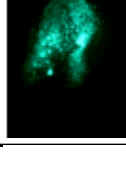
APPENDIX- 2

01-06-10	317	pCAT+ Hairy1	4-	14h	6			E		
	319		3+	14h	5+			E		
	320		4-	14h	6+		gs	PSM		
	321		3+	18h	6			PSM		
	322		4-	13h45	6?		gs	PSM		
	323		3	13h30	3+	x		PSM		
	324		4-	13h15	5			E		
	326		3+	16h30	5?		x	E		
	327		3+	16h40	5		late	A		


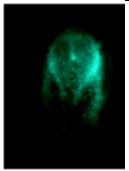

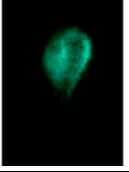

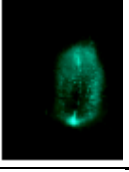

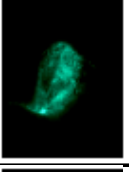

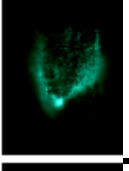



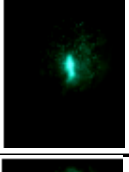

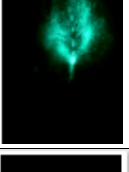

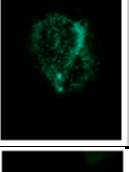

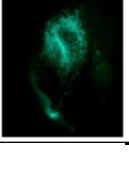
APPENDIX- 2

03-06-10	330	pCAT + Bra	4-	15h00	6?		gs		PSM		
	331		4	13h30	8+		+		PSM		
	332		4-	13h30	6?		x		P		
	333		4	13h	8+		+		PSM		
	334		4-	14h	5	x			PSM		
	335		3+	13h30	6?		x		PSM		
	336		4	13h30	5+		x		PSM		
	337		3+	12h	6++		gs		PSM		
	338		4-	14h45	6?	x			PSM		


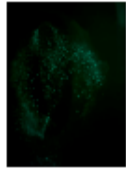

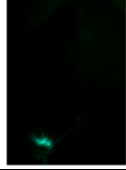

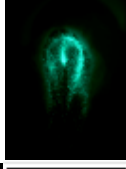

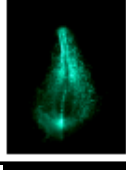

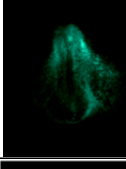

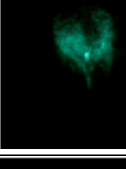

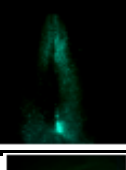

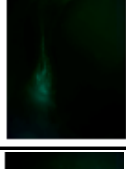

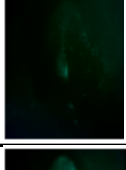

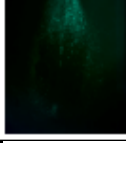
APPENDIX- 2

04-06-10	339	pCAT + Bra	3+	14h20	5		x		A		
	340		3+	13h	5		x		A		
	341		4-	13h	6++		gs		PSM		
	343		4-	12h45	6+		gs		PSM		
	344		3+	13h	4	x			PSM		
	345		4-	12h45	5?	x			PSM		
	346		4	13h20	6?		x		PSM		
	348		4+	13h30	8-				PSM		
	349		3+	22h	4		x		A		


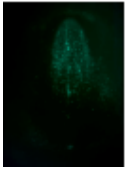

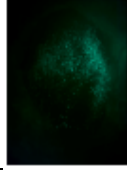

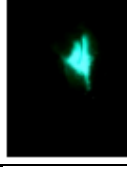



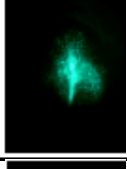

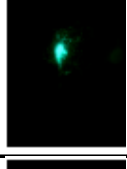

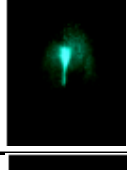



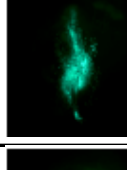

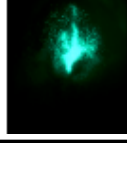
APPENDIX- 2

08-06-10	350	pCAT	3+	21h	5		late	A		
	352		4-	22h	5		late	A		
	353		3+	22h	5		late	E		
	354		3+	21h30	6?		x	PSM		
	355		3	22h	4+		late	P		
09-06-10	356	pCAT + Hairy	3+	18h	3+	x		PSM		
	357		3+	18h	4?	x		PSM		
	358		4-	17h	5		x	A		
	363		3+	22h	5		x	A		
	365		3+	22h	6		x	E		


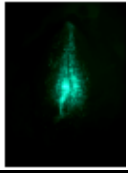

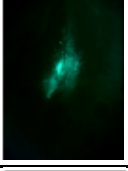

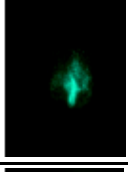

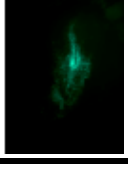
APPENDIX- 2

18-06-10	366	pCAT + Hairy	3+	22h	7+			A		
	367		3+	22h	6		late	P		
	368		4	21h40	5		late	A		
22-0610	370	pCAT + Hairy	3+	14h15	6++		gs	PSM		
	371		4-	21h	8?		gs	PSM		
	374		3+	20h30	5+		late	A		
	379		3+	20h30	8+		gs	PSM		
24-0610	387	pCAT + Bra	4	13h	8+			PSM		
	389		3+	18h30	8-			A		
	390		3+	18h10	8-			A		

APPENDIX- 2

	391		4-	18h	8-			A		
	392		4-	17h30	5		x	A		
25-0610	394	pCAT + Bra	4	14h	8?		gs	PSM		
	397		4-	18h	4-	x		PSM		
	398		4	13h	5+		x	PSM		
	401		4-	17h	5?		x	A		
	402		3+	17h	3+?	x		PSM		
	403		3+	11h40	8-		gs	PSM		
	404		4-	14h30	6++		gs	PSM		
	406		4-	14h30	6?		gs	PSM		

APPENDIX- 2

29-06-10	407	pCAT + Bra	4-	13h	6++		gs	PSM		
	409		4-	12h30	7++		gs	PSM		
	410		4	13h20	4?	x		PSM		
	412		4-	12h40	>6		gs	PSM		

P - Posterior; A - Anterior; E - Anterior and extraembryonic tissue; PSM - Presomitic mesoderm;
gs - phenotype with small number of somites; late - late growth